Protein Phosphorylation in Cyanobacterial Light-harvesting Complexes

A thesis submitted for the degree of Doctor of Philosophy

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Acknowledgement

It was my uncle, Mr. Alister Tsai who suggested that I came to Britain for my PhD studies three years ago. But for his suggestion and help, I would not be here and could not meet so many good friends.

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Declaration

All the results presented in this thesis were obtained by the author under the supervision of Dr. N. H. Mann, unless otherwise stated.

Experiments on the electrospray mass spectrometry of proteins were performed by Dr. A. Buzy in the Department of Chemistry. Experiments on the spectrophotometry of proteins were performed by the author and Dr. N. J. Silman in this laboratory.

All sources of information have been acknowledged by means of references. None of the work obtained in this thesis has been used for application for a degree.

Abbreviations:

ADP	Adenosine5'-diphosphate
APC	Allophycocyanin
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
Bis	N-N'-Methylene-bisacrylamide
Chl	Chlorophyll
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron)
DTT	Dithiothreitol
FPLC	Fast protein liquid chromatography
FSBA	5'-*p*-flourosulfonylbenzoyladenosine
HPLC	High pressure/performance liquid chromatography
IEF	Isoelectric focusing
LHC	Light-harvesting complex chlorophyll associated with photosystems
MES	2-[N-Morpholino]ethanesulfonic acid
NAD(P)	Nicotinamide adenosine dinucleotide (phosphate)
NAD(P)H	Nicotinamide adenosine dinucleotide (phosphate), reduced form
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer
PBS	Phycobilisomes
PC	Phycocyanin
PE	Phycoerythrin
PVDF	Polyvinylidene difluoride
PMSF	phenylmethylsulfonylfluoride
PS I (II)	Photosystem I (II)
SDS	Sodium dodecyl sulfate (or Sodium lauryl sulfate)
TEMED	(N,N,N',N'-Tetramethylethylenediamine)
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TLC	Thin layer chromatography
Tricine	N-tris(hydroxymethyl)methyl-glycine
Tris	Tris(hydroxymethyl)methylamine

Summary

Protein kinase activities have been demonstrated in the thylakoid membranes of higher plants and in cyanobacteria, furthermore, their role in state-transitions has been widely accepted. Several proteins in cell-free extracts of *Synechocystis* sp. PCC 6803 were found to be phosphorylated *in vitro* and the existence of protein kinase activities was thus demonstrated. Total membranes, obtained by pelleting the cell-free extracts, were subjected to sucrose density gradient centrifugation to separate the thylakoid membranes. The thylaoid membranes were found in the 50 to 60% sucrose region. Two blue bands located in the 30% sucrose fraction contained phycobiliproteins. *In vitro* phosphorylation analysis showed that several proteins were strongly phosphorylated, especially an 18 kDa protein, which occurred in both the phycobiliprotein-containing fractions of a sucrose density gradient and the thylakoid membranes was strongly phosphorylated.

Digestion of the sucrose density gradient- and SDS-PAGE-purified, labelled 18 kDa protein with Staphylococcal V8 protease yielded a labelled 9 kDa fragment, which was the N-terminal part of the 18 kDa protein. The 18 kDa protein was tentatively identified as β-phycocyanin by comparison of the N-terminal amino acid sequence of the 9 kDa polypeptide against proteins in the protein databases. The phosphoamino acid of β-phycocyanin was identified as phosphoserine by acid hydrolysis of SDS-PAGE-purified β-phycocyanin. However, which particular serine residue(s) which is modified is still to be determined.

Besides protein kinase activities, phosphatase activities were also demonstrated by pulse-chase reactions. Phosphorylation and dephosphorylation of β-phycocyanin occurred simultaneously and were insensitive to classical inhibitors of protein kinases and phosphatases. β-phycocyanin, β-phycocyanin kinase and the phosphatase co-occurred and could not be separated by anion exchange chromatography and gel filtration chromatography, suggesting that β-phycocyanin and β-phycocyanin kinase were located in a macromolecular complex. Denaturation of the β-phycocyanin complex by urea showed that β-phycocyanin kinase activities could be denatured and renatured. However, β-phycocyanin kinase activity could not be restored after separation of the denatured β-phycocyanin complex by anion exchange chromatography in the presence of urea.

Phosphorylation and dephosphorylation of β-phycocyanin were light-independent *in vitro*. Spectroscopy showed that the absorption spectra of β-phycocyanin before and after phosphorylation were not significantly altered. However, phosphorylation of β-phycocyanin resulted in fluorescence quenching, suggesting there was a loss of excitation energy, which might be in the form of non-radiative dissipation.

Screening of cosmid library containing *Synechocystis* sp. PCC 6803 genome showed that a cosmid clone contained *Synechocystis* sp. PCC 6803 *cpcB* gene, encoding β-phycocyanin. The predicted amino acid sequence deduced from the *Synechocystis* sp. PCC 6803 *cpcB* gene provided further evidence that the 18 kDa protein was β-phycocyanin.

Chapter 1

Introduction

1.1. Cyanobacteria

Cyanobacteria, photosynthetic and oxygen-evolving prokaryotes, have affected the earth's environment since the Pre-Cambrian era. Like green plants and eukaryotic algae, cyanobacteria possess two photosystems, serving the function of photosynthesis. The evolution of oxygen and removal of CO_2 by cyanobacteria also make a fundamental contribution to the biosphere. Cyanobacteria synthesise chlorophyll *a* as well accessory light-harvesting pigments, phycobiliproteins, which comprise varying proportions of phycoerythrin, phycocyanin and allophycocyanin. The blue or red colouration of these pigments contribute to the organisms appearance and hence they used to be called "blue-green algae". Cyanobacteria are probably the most diverse group of prokaryotes and are found in a wide range of habitats. These range from the polar regions to hot springs. There are several reviews that describe the ecology, physiology, biochemistry and molecular biology of these organisms (Fogg *et al.*, 1973; Carr and Whitton, 1982; Stanier and Cohen-Bazire, 1977; Fay and Van Baalen, 1987; Mann and Carr, 1992; Bryant, 1995). Cyanobacteria show a great diversity in DNA base compositions with a G+C content ranging from 34 to 71% (Herdman *et al.*, 1979). As distinct from other known prokaryotes, cyanobacteria also show a wide range of genome sizes, ranging from $1.6x10^9$ to $8.6x10^9$ daltons, this later being one of the largest prokaryotic genomes that has been determined (Herdman *et al.*, 1979). Apart from the diversity shown in cyanobacteria habitats and DNA compositions, cyanobacteria also show a diversity of morphological forms. These vary from the simple unicellular forms to the more complicated branching filamentous forms of cyanobacteria. The simplest forms are demonstrated by the unicellular cyanobacteria that divide by binary fission or by budding. Other unicellular cyanobacteria produce baeocytes, small cells which arise from multiple fission of a vegetative cell. The other main subgroups of cyanobacteria are filamentous in

form and some of these show a number of differentiated cell types. Some filamentous forms can produce hormogonia, which are short motile chains of smaller cells than the vegetative trichome. Hormogonia are usually produced under specific conditions, such as when the organism has been inoculated into fresh medium. Certain filamentous cyanobacteria produce differentiated cells, heterocysts when they are transferred to medium limited in fixed nitrogen and these occur at regular intervals. Some of the heterocystous cyanobacteria also produce additional differentiated cell types, akinetes, which, like spores, tend to be more resistant to adverse conditions, particularly cold and desiccation, than vegetative cells.

1.1.1. Taxonomy of the cyanobacteria

Phylogenetic studies based on the nucleotide sequences of rRNAs and photosynthesis-related genes of cyanobacteria and chloroplasts, and the gene encoding ribosomal protein S1 from *Synechococcus* sp. PCC 6301 have provided evidence supporting the well-accepted endosymbiosis hypothesis (Sugita *et al.*, 1995; for review see Woese, 1987). Comparison of the 16S rRNAs from 29 cyanobacteria and that from chloroplasts supports the idea that cyanobacteria and green chloroplasts form a coherent phylogenetic group and also suggests that the chloroplast lineage is contained within the cyanobacterial radiation (Giovannoni *et al.*, 1988). Cyanobacteria are classified into the Gram-negative eubacteria on the basis of comparison of their nucleotide base sequence data of 16S and 5S rRNA with that of the other phyla of eubacteria (for review see Fox *et al.*, 1980 and Woese, 1987). Besides, cyanobacteria possess typical Gram-negative cell envelopes: a peptidoglycan layer is found between the outer and the inner membranes (for review see Darnell *et al.*, 1986 and Golecki *et al.*, 1982). Some botanists, however, insist that cyanobacteria should be classified into the algae of the plant kingdom in that

cyanobacteria possess two photosystems and are oxygenic cells (Goodwin and Mercer, 1983). Even so, the term "cyanobacteria" is used throughout this study.

1.1.2. Nutritional modes of the cyanobacteria

Cells may be classified as autotrophs and heterotrophs in terms of their nutritional mode. Cells that can produce their organic molecules from primarily CO_2 are autotrophs. Those cells which grow and maintain themselves by taking up extrinsic reduced organic carbon sources are heterotrophs. The primary nutritional mode of cyanobacteria is photoautotrophy. Like the photosynthetic cells of green plants, cyanobacteria can capture light of a certain spectral quality and make use of the light energy to drive the formation of ATP and NADPH used for the assimilation of CO_2. More than half of the cyanobacteria tested are capable of facultative photoheterotrophy, of which 15-20% are also able to grow under chemoheterotrophic conditions (for review see Tandeau de Marsac and Houmard, 1993). When cyanobacteria are transferred to the dark from photoautotrophic conditions, they can utilise glucose derived from glycogen through the oxidative pentose phosphate pathway (Figure 1.1). Some strains of cyanobacteria are capable of photoheterotrophic growth at the expense of glucose or fructose as the reduced organic carbon source (for review see Sherman *et al.*, 1987). *Synechocystis* sp. PCC 6803, the organism on which this study is based, exhibits light-activated heterotrophic growth (LAHG), that is, *Synechocystis* sp. PCC 6803 can grow in the dark, consuming exogenous glucose as long as it is illuminated with blue light for 5 min in every 24 hr period (Anderson and McIntosh, 1991).

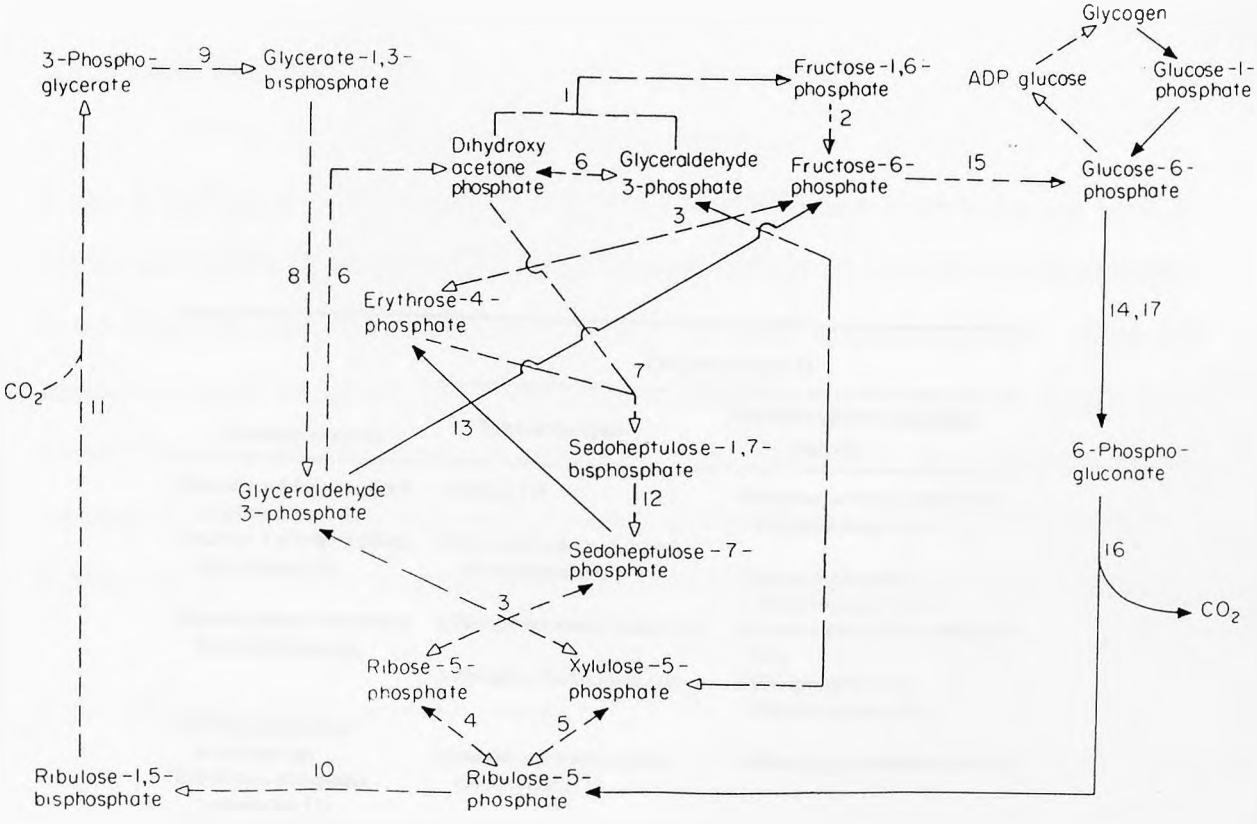

Fig. 1.1. Reactions specific to CO_2 fixation, the Calvin cycle (— — —), to carbohydrate catabolism, the oxidative pentose phosphate pathway (———) and common to both (— - - —). To identify the enzymes of the Calvin cycle and the oxidative pentose pathway refer to Table 1.1.

(Adapted from Smith, 1982).

Common enzymes	Enzymes unique to:	
	The Calvin cycle	Oxidative pentose phosphate pathway
Fructose-1,6-bisphosphate aldolase (1)	Aldolase (7)	Dihydroxyacetone transferase (transaldolase) (13)
Fructose-1,6-bisphosphate phosphatase (2)	Glyceraldehyde 3-phosphate dehydrogenase (8)	Glucose 6-phosphate dehydrogenase (14)
Glycoaldehyde transferase (transketolase) (3)	3-Phosphoglycerate kinase (9)	Hexose-6-phosphate isomerase* (15)
	5-Phosphoribulokinase (10)	6-Phosphogluconate dehydrogenase (16)
Ribose-5-phosphate isomerase (4)	Ribulose-1,5-bisphosphate carboxylase (11)	6-Phosphogluconolactonase (17)
Ribulose-5-phosphate 3-epimerase (5)		
Triose phosphate isomerase (6)	Sedoheptulose-1,7-bisphosphate phosphatase (12)	

Table. 1.1. Enzymes of the Calvin cycle and the oxidative pentose phosphate pathway. The numbers in parentheses identfy the reactions in Fig. 1.1. that catalysed by these enzymes. (Adapted from Smith, 1982).

1.2. Photosynthesis

Photosynthesis is a series of processes by which photosynthetic cells capture the light to produce carbohydrate from CO_2 and H_2O. Photosynthesis consists of two series of chemical reactions, light reaction and the dark reaction, which will be detailed in 1.2.1. and 1.2.3., respectively.

1.2.1. Light reaction

Light is defined as electromagnetic radiation which possesses both wave and particle attributes. Light with wavelengths falling between 400 nm and 700 nm is called visible light. Different spectral qualities of light will, in terms of photosynthesis, affect the excitation energy transfer and phosphorylation and dephosphorylation of proteins through the activation of the activities of protein kinase(s)/phosphatase(s). The light reaction is a consecutive and complicated series of reactions by which photosynthetic pigments harvest the light to drive the synthesis of ATP and NADPH, used for the fixation of CO_2.

1.2.2. Pigments and absorption spectra

Pigments involved in the light reaction of photosynthetic cells are classified into three classes, namely, chlorophylls, carotenoids and phycobilins (Goodwin and Mercer, 1983). All the three kinds of pigments occur as chromoproteins, that is, they are pigment-protein complexes. The linkages between carotenoids and proteins, and between chlorophylls and proteins are non-covalent bonds and, therefore, carotenoids and chlorophylls can be easily extracted from photosynthetic cells using acetone or alcohol. In contrast, the linkages between phycobilins and proteins are covalent bonds and thus phycobilins in photosynthetic cells are referred to as phycobiliproteins and the phycobilins must be

isolated by more rigorous methods. Phycocyanobilin, for example, can be obtained from phycocyanin or allophycocyanin by methanolysis (Beuhler *et al.*, 1976). Four kinds of chlorophylls have been found in different kinds of photosynthetic cells, termed chlorophyll *a*, *b*, *c* and *d*. Cyanobacteria contain only chlorophyll *a*. Many kinds of carotenoids are known, among which echinenone, cathaxanthin, caloxanthin and nostaxanthin occur in cyanobacteria (for review see Sandmann, 1994). In addition to chlorophylls and carotenoids, phycobiliproteins can also be found in red algae and cyanobacteria. Four main phycobilins have been found in red algae and cyanobacteria: phycocyanobilin, phycoerythrobilin, phycobiliviolin and phycourobilin (for review see Glazer, 1989). The structures of these four phycobilins are shown in Figure 1.2. Phycobilins are bound to proteins through thioester bonds to form phycobiliproteins (for review, see Glazer, 1992 and Grossman *et al.*, 1993). Phycobiliproteins are assembled into phycobilisomes via linker polypeptides with molecular masses ranging from 8 to 120 kDa. Phycobilisomes and phycobiliproteins will be described in detail in 1.3.2. The roles that chlorophylls, carotenoids and phycobiliproteins play in the light reaction of photosynthesis are to absorb the visible light and pass the excitation energy to reaction centres (for review, see Goodwin and Mercer, 1983; Sidler, 1995; Hirschberg and Chamovitz 1995).

1.2.3. Photosystems and reaction centres

Like green plants, cyanobacteria possess two photosystems; they are photosystem I and photosystem II. Photosystem II (PS II) is defined as a light-driven water : plastoquinone oxidoreductase, while photosystem I (PS I) is a light-driven plastocyanin : ferredoxin oxidoreductase. PS II and PS I are connected by cytochrome b_6/f, which acts as a plastoquinone : plastocyanin reductase (for review see Gregory, 1989; Barry *et al.*, 1995; Kallas, 1995 and Golbeck, 1995). Both of the two photosystems consist of

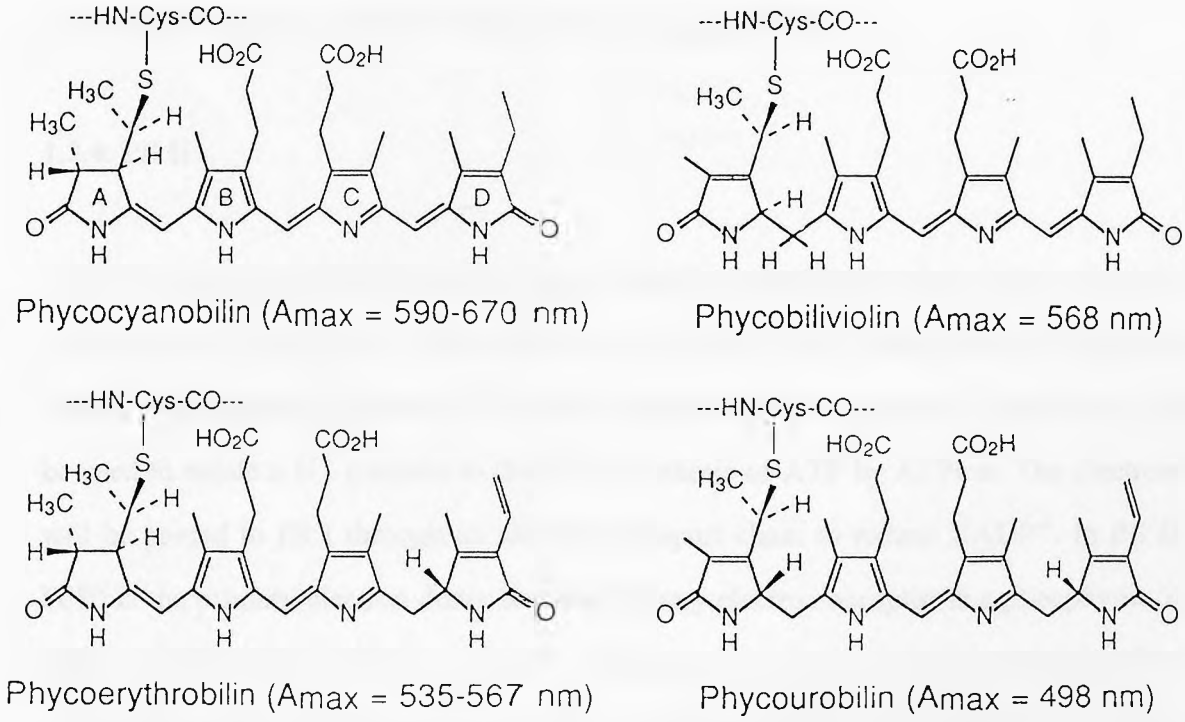

Fig. 1.2. The structures of phycobilins and their peptide linkage in phycobiliproteins. The linkages presented here are between ring A and a cysteine residue of the phycobiliprotein, but phycoerythrobilin and phycourobilin chromophores can be linked to the apoprotein via both ring A and D (Grossman *et al.*, 1993).

pigment antennae and reaction centres. The reaction centres of PS I and PS II are referred to as P680 and P700. When the light is captured by the pigment antennae the energy is passed to the reaction centre in which the chlorophyll *a* molecules become excited and consequently release electrons. The oxidised chlorophyll *a* molecules in PS I reaction centre can then be re-reduced by the electrons coming from PS II reaction centre through an electron transport chain, which links PS I and PS II, while the oxidised chlorophyll *a* molecules in PS II reaction centre are re-reduced by the electrons coming from H_2O (for review see Rutherford, 1989; Golbeck, 1992; Vermaas, 1993).

1.2.4. PS II

PS II is a membrane-bound protein complex which possesses the water splitting activity (for review see Rutherford, 1989; Matto *et al.*, 1989). During photosynthesis the water molecule is split into protons and electrons and the oxygen is released. The protons will be used to create a H^+ gradient to drive the synthesis of ATP by ATPase. The electrons will be passed to PS I through an electron transport chain to reduce $NADP^+$. In PS II, P680 is the primary electron donor and the primary electron acceptor is a pheophytin. A tightly bound plastoquinone molecule, referred to as Q_A, accepts electrons from pheophytin, and in turn passes the electrons to another plastoquinone, Q_B, which accepts two consecutive electrons from Q_A. PS II core proteins are composed of six major polypeptides. They are a 32 kDa polypeptide (also called the D1 polypeptide), which is the location of Q_B, a 47-51 kDa polypeptide, a 43-45 kDa polypeptide, another 32 kDa (also called the D2 polypeptide), which is the location of Q_A, a 10 kDa polypeptide, which is cyt$_{b559}$ and a 4.5 kDa polypeptide. The six polypeptides are encoded by *psbA, psbB, psbC, psbD, psbE,* and *psbF,* respectively (for review see Stewart, 1988). There is evidence suggesting that PS II might exist as dimer *in vivo* in *Synechocystis* sp. PCC 6803 (Boekema *et al.*, 1995) The structure of PS II is shown in Figure 1.3.

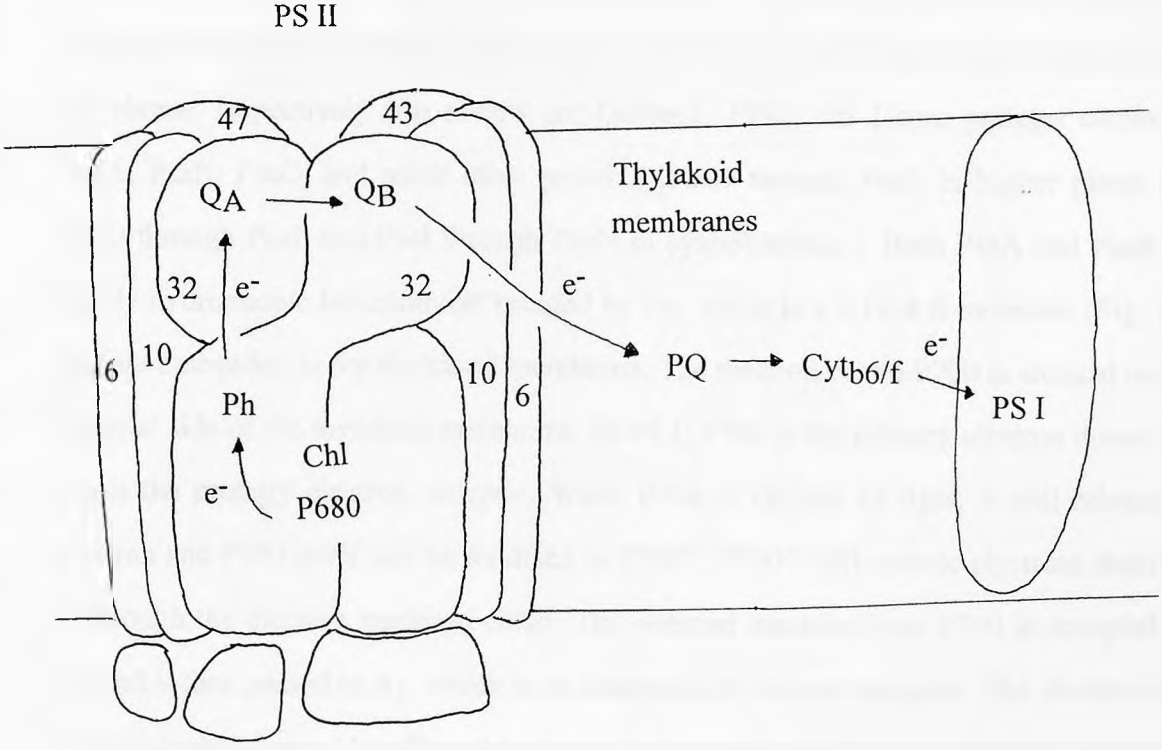

Fig. 1.3. The structure of photosystem II. The molecular masses are marked on each polypeptide. Polypeptides spanning the membrane are the PS II core proteins. P680, after capturing light energy, will release an electron and becomes the oxidised form, $P680^+$. The electron will be received by a pheophytin. The pheophytin sequentially passes the electron to Q_A, to Q_B and then to PS I through an electron transport chain. $P680^+$ will be re-reduced by receiving an electron released from H_2O.

Abbreviations: Ph, pheophytin; Cyt, cytochrome; Chl, chlorophyll; PQ, the plastoquinone pool.

1.2.5. PS I

Photosystem I contains plastocyanin : ferredoxin oxidoreductase activity. It occurs as a membrane-bound pigment-protein complex in the thylakoid membrane in higher plants, algae and cyanobacteria. There is evidence showing that PS I is a protein heterodimer (Krauß et al., 1993). Plastocyanin and ferredoxin are physically separated by the thylakoid membrane and are located on the lumenal side and stromal side of the thylakoid membrane, respectively (for review see Golbeck, 1992). PS I core proteins consist of PsaA, PsaB, PsaC, and some other proteins (PsaD through PsaL in higher plants and PsaD through PsaF and PsaI through PsaN in cyanobacteria). Both PsaA and PsaB are highly hydrophobic heterodimers bridged by Fx, which is a 4 Fe-4 S molecule (Fig. 1.4) and are embedded in the thylakoid membrane. The reaction centre P700 is situated on the lumenal side of the thylakoid membrane. In PS I, P700 is the primary electron donor and A_0 is the primary electron acceptor. When P700 is excited by light, it will release an electron and P700 itself will be oxidised to $P700^+$. $P700^+$ will receive electrons from PS II through the electron transport chain. The released electron from P700 is accepted by A_0 and is then passed to A_1, which is an intermediate quinone acceptor. The electron will then be passed from A1 to Fx, which is a 4Fe-4S cluster and links both PsaA and PsaB proteins together. Fx then passes the electron to F_A/F_B which are two 4Fe-4S clusters and reside in PsaC. The electron is finally passed to ferredoxin with the cooperation of PsaD. The structure of PS I reaction centre is shown in Figure 1.4.

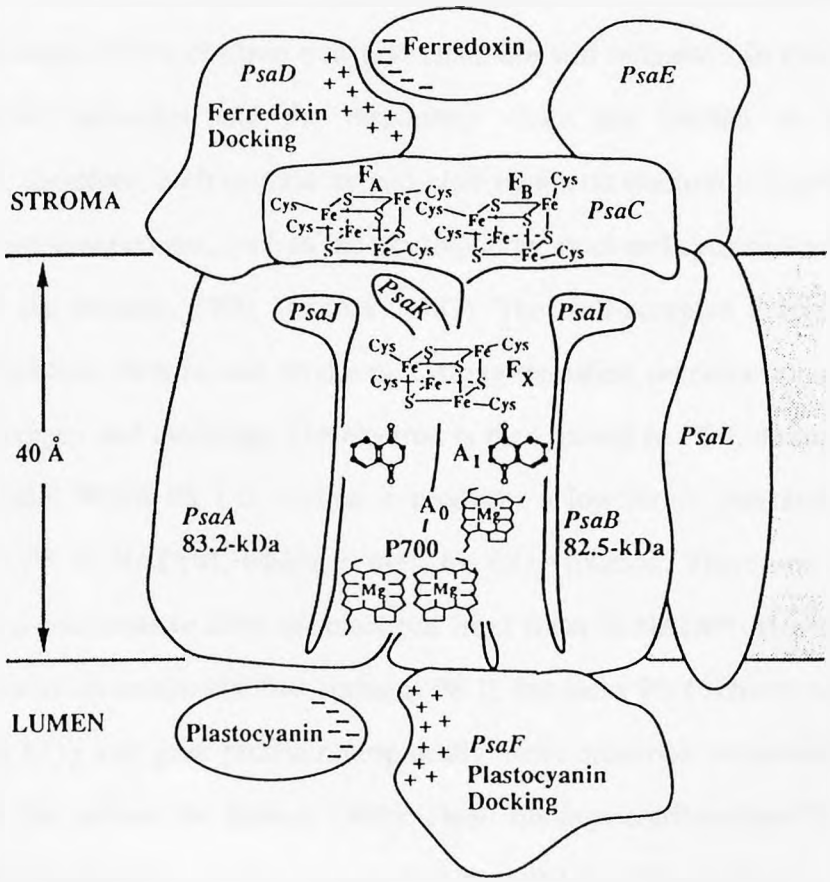

Fig. 1.4. The proposed structure of photosystem I (adapted from Golbeck 1992). The PsaC, PsaD and PsaE polypeptides are located in the stromal side of the membrane. The PsaF polypeptide is located in the lumenal side of the membrane. The PsaI, PsaJ, PsaK, and PsaL polypeptides span the membrane. The PsaA, PsaB and PsaC are the PS I core proteins. There are two phylloquinones molecules which are assumed to be distributed between the PsaA and PsaB proteins. P700 is located on the lumenal side of the membrane, and the plastocyanin and the ferredoxin molecules are physically separated by the membrane.

1.2.6. Photosynthesis and electron transport chain

PS II and PS I are linked together by an electron transport chain. Even though cyanobacteria, like higher plants, perform photosynthesis and possess two photosystems, some components of the electron transport chain are still unknown. In cyanobacteria the photosynthetic apparatus and the respiratory chain are located in the thylakoid membranes, therefore, both respiratory and photosynthetic electron transport chains may share common components, such as the plastoquinone pool and cytochrome *b6/f* complex (for review see Scherer, 1990, Peschek, 1987). The well-accepted Z-scheme describes how PS II absorbs photons and produces a strong oxidation potential to split water into molecular oxygen and electrons. The electron is then passed to PS I, through an electron transport chain. When PS I is excited it produces a low redox potential necessary to reduce $NADP^+$ to NADPH, which is used for CO_2 fixation. Therefore, two different photosystems cooperate to drive the electrons from water to $NADP^+$. However, a mutant of *Chlamydomonas reinhardtii* that contains PS II, but lacks PS I activity can still reduce $NADP^+$, fix CO_2 and grow photoautotrophically under anaerobic condition (Greenbaum *et al.*, 1995, for review see Barber, 1995). These findings confirm that PS II alone can support photoautotrophic growth by generating NADPH for CO_2 fixation.

1.2.7. Dark reaction

Fixation of CO_2 to produce carbohydrate doesn't directly require light energy and is hence termed the dark reaction. In order to biosynthesise glucose, CO_2 must be assimilated. The concentration of CO_2 is only 0.03% in air and fixation of CO_2 is dependent on the concentration of the internal dissolved inorganic carbon (DIC) (for review see Crotty *et al.*, 1994).

1.2.8. Mechanism of inorganic carbon uptake

Many photosynthetic microorganisms possess an inorganic carbon concentrating mechanism (Ci-concentrating mechanism), which enables them to accumulate Ci for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (for review see Kaplan *et al.*, 1991). The activity of the Ci-concentrating mechanism increases when these photosynthetic microorganisms adapt to low from high external CO_2 concentration (Kaplan *et al.*, 1991).

1.2.9. CO_2 fixation

The Calvin cycle is a series of biochemical reactions by which photosynthetic organisms produce glucose from CO_2 and H_2O. The overall reaction of Calvin cycle converts 6 molecules of CO_2 into 1 molecule of fructose-6-phosphate at the expense of 12 molecules of NADPH and 18 molecules of ATP. Fructose-6-phosphate will then be sequentially converted into glucose-6-phosphate, glucose-1-phosphate and glucose by different enzymes. The Calvin cycle is shown in Figure 1.1.

1.3. Light-harvesting complexes

The function of light-harvesting complexes is common to all photosynthetic organisms. Possessing two photosystems, however, cyanobacteria and green plants have different components and structures of light-harvesting complexes, serving the same function in capturing the light.

1.3.1. Chlorophyll *a/b* pigment proteins associated with photosystems

The primary light-harvesting complexes in green plants are chlorophyll *a/b* pigment proteins associated with photosystems (LHC), among which the LHC associated with PS II (LHC II) is widely studied. LHC II contains a number of closely-related light-harvesting polypeptides, which are encoded by nuclear *cab* genes, whose genomic DNA and cDNA have been sequenced from many green plant species (for review see Bennett, 1991; Allen, 1992).

1.3.2. Phycobilisomes (PBS)

The light-harvesting complexes in cyanobacteria are phycobilisomes (PBS). Cyanobacteria, with the exception of the eukaryotic red algae, are the only known photosynthetic organisms that possess such structures. Phycobilisomes are mainly composed of phycobiliproteins, which constitute up to half of the soluble proteins in the cell. Phycobiliproteins are brilliantly-pigmented, water-soluble proteins (for review see Grossman *et al.*, 1993a and 1993b). The major phycobiliproteins in cyanobacteria are phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC). Some uncoloured polypeptides, linker polypeptides, are indispensable for PBS assembly. All cyanobacteria contain APC and PC. Allophycocyanin-B (APC-B) has also been found in some cyanobacteria. PEs are widely distributed among all groups of cyanobacteria, but are not universally present (for review see Rippka *et al.*, 1979; Holt *et al.*, 1994). During photosynthesis, when the light is absorbed by the phycobiliproteins the excitation energy which is absorbed by PE is sequentially transferred to PC, to APC and then to PS II reaction centres. Under some conditions, the excitation energy might be transferred from phycobiliproteins to PS I (Su *et al.*, 1992).

1.3.3. Phycobiliprotein genes

PE, PC and APC are the major phycobiliproteins in cyanobacteria. Each phycobiliprotein is composed of a trimer of both α and β subunits, two trimers being stacked together to form a hexamer. Recently phycoerythrin, phycocyanin and allophycocyanin have been isolated and crystallised (Schirmer *et al.*, 1985; Fincer *et al.*, 1992; Brejc *et al.*, 1995). Both α and β subunits of a given phycobiliprotein are composed of eight helices called X, Y, A, B, E, F, G and H, and they are connected by some irregular loops (Schirmer *et al.*, 1985; Brejc *at al.*, 1995). The (αβ) monomer, $(\alpha\beta)_3$ trimer and $(\alpha\beta)_6$ hexamer of a given phycobiliprotein are formed through hydrophobic, polar and electrostatic interactions between the α and β subunits (Schirmer *et al.*, 1985 and 1986; Brejc *et al.*, 1995). The globular part (helices A to H) of each subunit structure closely resembles the globin fold. The site of attachment of chromophore (bilin groups) β84, as well as α84, is topologically equivalent to the attachment of the haem via the distal histidine (His E7) of myoglobin (Schirmer *et al.*, 1985). The structures of monomer and trimer of both α and β subunits of C-phycocyanin are shown in Fig. 1.5. The α and β genes coding for α and β subunits of a given phycobiliprotein are always contiguous and cotranscribed (for review see Grossman *et al.*, 1993a and 1993b). Additionally, linker polypeptides are indispensable for the assembly of phycobilisomes. Table 1.2 shows the genes, subunits and chromophore contents of phycobiliproteins.

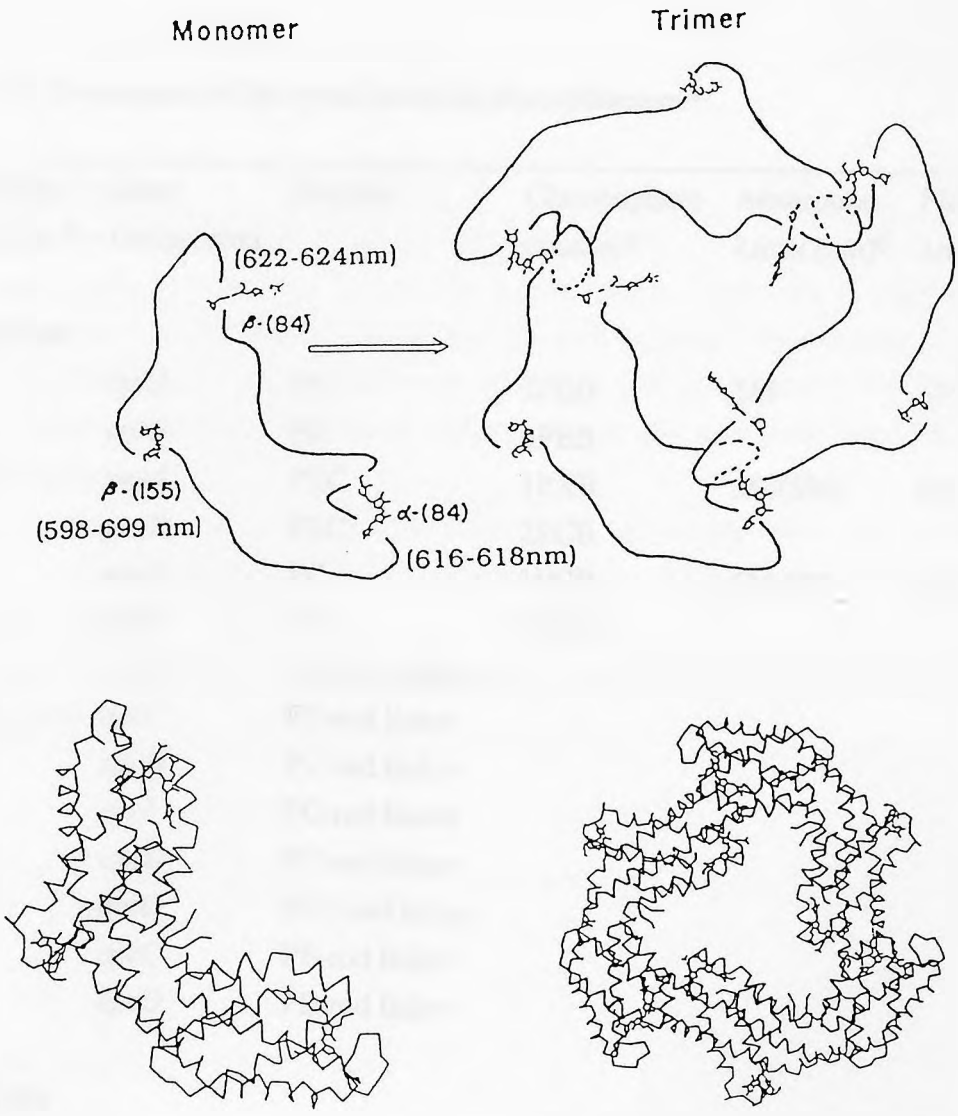

Fig. 1.5. The structures of a monomer and a trimer of both α and β subunits of C-phycocyanin. (a) Schematic structure of a monomer and a trimer of C-phycocyanin (adapted from Mimuro *et al.*, 1986). (b) Trimeric structure of C-phycocyanin derived from the X-ray diffraction structure (adapted from Schirmer *et al.*, 1985, 1987).

Table 1.2. Components of the cyanobacterial phycobilisomes[a]

Polypeptide designation [b]	Gene Designation	Protein	Chromophore content [c]	Absorption λmax (nm)[d]	Fluorescence λmax (nm) [d]
Rod Components					
α^{PE}	*cpeA*	PE	2PEB	565	575
β^{PE}	*cpeB*	PE	3PEB		
α^{PEC}	*pecA*	PEC	1PXB	568(590)	625
β^{PEC}	*pecB*	PEC	2PCB		
α^{PC}	*cpcA*	PC	1PCB	615-620	625-645
β^{PC}	*cpcB*	PC	2PCB		
L_{RC}	*cpcG*	rod-core linker			
L_R^{PC}	*cpcC*	PC-rod linker			
	cpcH	PC-rod linker			
	cpcI	PC-rod linker			
	cpcD	PC-rod linker			
LRPEC	*pecC*	PEC-rod linker			
LRPE	*cpeC*	PE-rod linker			
	cpeD	PE-rod linker			
Core components					
α^{AP}	*apcA*	APC	1PCB	650	660
β^{AP}	*apcB*	APC	1PCB		
α^{APB}	*apcD*	APC B	1PCB	670	675
$\beta^{18.3}$	*apcF*	18.3 subunit	1PCB	616	640
L_{CM}	*apcE*	core-membrane linker	1PCB	665	680
L_C	*apcC*	core linker			

(continued)

Table 1.2. (continued)

a	Adapted from Tandeau de Marsac and Houmard (1993)
b	α and β refer to the α and β chain of a given phycobiliprotein; L represents linker polypeptides. The subscripts and superscripts to L represent the locations of the linker polypeptides and the phycobiliproteins to whch linker polypeptides link, respectively. The superscripts to any symbol or alphabet means the molecular mass of that given polypeptide.
c	Abbreviations: Linker, linker polypeptides; PCB, phycocyanobilin; PC, phycocyanin; PEB, phycoerythrobilin; PE, phycoerythrin; PXB, phycobiliviolin; APC, allophycocyanin.
d	absorption and fluorescence maxima depend slightly on the association with linker polypeptides.

1.3.4. Biosynthesis of phycobiliproteins

What the mechanisms of chromophore biosynthesis are and how chromophores attach to apoproteins to form phycobiliproteins are poorly documented and not fully elucidated. Beale and Cornejo (1991a, 1991b and 1991c) proposed the pathway of phycobilin biosynthesis, which is shown in Figure 1.6. Two gene products, CpcE and CpcF, from *Synechococcus* sp. PCC 7002 exhibit the phycocyanobilin lyase specific for the Cys-84 position of the α-subunit phycocyanin (α-phycocyanin). Inactivation of *cpcE* and *cpcF* genes together or separately by insertion resulted in the attachment of phycocyanobilin not exclusive to Cys-84 in the α phycocyanin (Fairchild *et al.*, 1992). Expression of α and β phycocyanin in red alga *Cyanidium caldarium* shows that the *cpcBA* transcript, coding for β and α phycocyanin, can be detected in the light, or in the dark as long as *C. caldarium* is incubated with 10^{-6} M heme (Troxler *et al.*, 1995). Further experiments also show that heme is involved in the light induction of the *cpcBA* operon (Troxler *et al.*, 1995).

1.3.5. Morphology of phycobilisomes

Phycobiliproteins are integrated into phycobilisomes via linker polypeptides. Phycobiliproteins extracted from cyanobacteria are acidic proteins, whereas all the linker polypeptides are basic. Hence, it has been suggested that electrostatic interactions between the linker polypeptides and the phycobiliproteins could stabilise the PBS assembly (for review see Sidler, 1995). Several lines of evidence also support the notation that linker polypeptides are indispensable for the phycobilisome assembly in *Synechococcus* sp. PCC 7002 (de Lorimier *et al.*, 1990a and 1990b) and in *Synechococcus* sp. PCC 7942 (Bhalerao *et al.*, 1993). Experiments also show that genes encoding linker polypeptides and their corresponding phycobiliproteins in *Synechococcus*

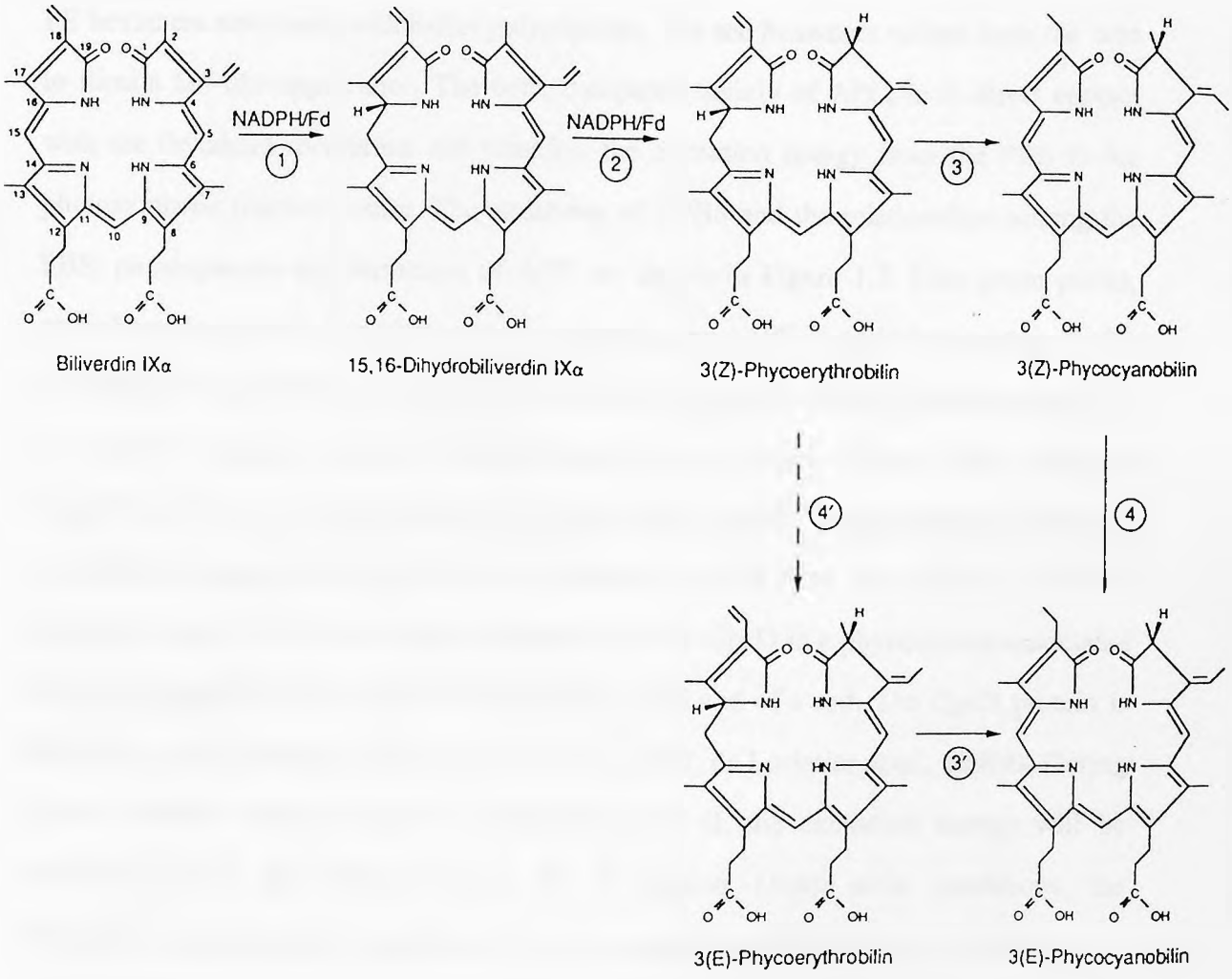

Fig. 1.6. The proposed biosynthetic pathway of phycobilins (Adapted from Beale and Cornejo, 1991c). Biliverdin IXα is formed from the heme group through the heme oxygen system (Beale and Cornejo, 1984). It has been identified that 3(Z)-phycoerythrobilin and 3(Z)-phycocyanobilin are other intermediates in the conversion of biliverdin IXα to 3(E)-phycocyanobilin (Beale and Cornejo, 1991b). Enzyme in reaction 1 is ferredoxin-$NADP^+$ reductase; however, enzymes in the other reactions are currently unknown.

sp. PCC 6301 (Houmard *et al.*, 1986), *Calothrix* sp. PCC 7601 (Federspiel and Scott, 1992; Lomax *et al.*, 1987; Conley *et al.*, 1988) and *Anabaena* sp. PCC 7120 (Belknap and Haselkorn, 1987) are clustered in the genomes and may be cotranscribed. Phycobilisomes consist of two substructures, the core and the rods. The rods are comprised of PC and/or PE hexamers associated with linker polypeptides. The rod hexamers radiate from the core to form a fan-like appearance. The core, composed mainly of APC, is in direct contact with the thylakoid membrane and transfers the excitation energy from the PBS to the photosynthetic reaction centre. The structures of a PBS and the relationships among the PBS, photosystems and formation of ATP are shown in Figure 1.7. Like green plants, cyanobacteria possess two photosystems, photosystem I (PS I) and photosystem II (PS II). Both PS I and PS II are located in the thylakoid membrane and are linked together by an electron transport chain. Phycobilisomes are in direct contact with thylakoid membrane. There is evidence showing that ferredoxin-$NADP^+$ oxidoreductase (FNR) has a CpcD-like domain in its N-terminus in *Anabaena* sp. PCC 7120 (Lomax *et al.*, 1987; de Lorimier *et al.*, 1990a; Schluchter and Bryant, 1992). CpcD is a phycocyanin-associated linker polypeptide which is located at the core-distal end of a rod. The CpcD protein is believed to terminate the PBS rod (Lomax *et al.*, 1987; de Lorimier *et al.*, 1990a). During photosynthesis, when the light is absorbed by PS II, the excitation energy will be transferred from phycobiliproteins to PS II reaction. Under some conditions, the excitation energy might be transferred from phycocyanin to PS I (Su, *et al.*, 1992).

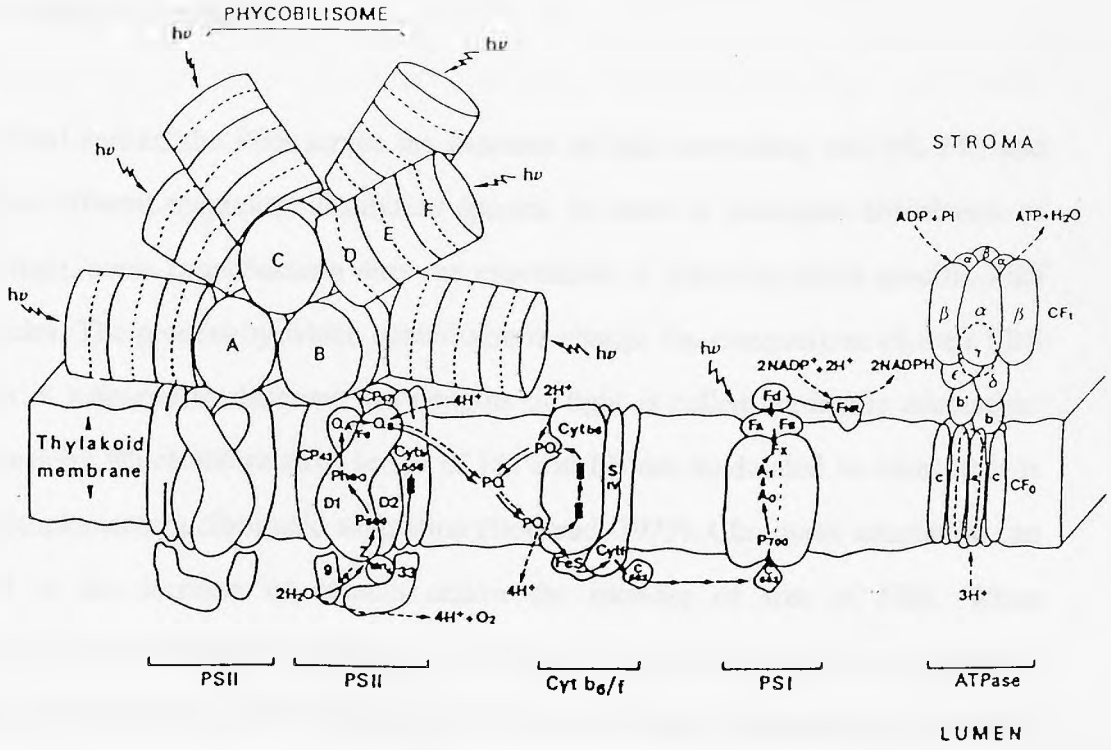

Fig. 1.7. The photosynthetic apparatus of a typical cyanobacterium (Adapted from Tandeau de Marsac, N. 1991).

The thylakoid membranes accommodate PS II, which exists as a dimer and PS I core proteins, cytochrome $b_{6/f}$ complex, the CF_0 subunit of the ATPase and the plastoquinone pool, which disperses in the thylakoid membranes. Some other PS II and PS I proteins are either located in the lumenal side or the stromal side of the thylakoid membranes. The CF_1 of the ATPase protrude to the stromal side of the thylakoid membranes. The phycobilisome is in direct contact with the thylakoid membranes. Routes of the electron (—) and of the H^+ (---) are shown in the diagram. When P680 releases an electron, the electron is sequentially passed to the pheophytin molecule, to QA, to QB and then to the plastoquinone pool, which then passes the electrons to the cytochrome $b_{6/f}$ complex and then to PS I. The plastoquinone also accepts the H^+ from the stroma of the thylakoid membranes and releases the H^+ to the lumen. The H^+ in the lumen then creates a H^+ gradient is used to drive the formation of ATP by the ATPase. The H^+ in the stroma can be used to form the NADPH by FNR.

Abbreviations: Cyt, cytochrome; PQ, the plastoquinone pool; FNR, ferredoxin:$NADP^+$ reductase.

1.3.6. Chromatic adaptation

As described earlier, the PBS serves the function of light harvesting and PE, PC, and APC have different maximum absorption spectra. In order to maximise absorbance of incident light, some cyanobacteria alter the expression of genes encoding specific PBS polypeptides. The process by which cyanobacteria change the composition of their PBS structures in response to different wavelengths of light is called chromatic adaptation. The process by which the relative levels of PC and PE are modulated to each other is termed complementary chromatic adaptation (Bogorad, 1975). Chromatic adaptation can also lead to the increase of amount and/or the increase of size of PBS. When *Synechococcus* sp. PCC 6301 is shifted to low and red light after being grown in high and white light, both the size and the amounts of PBS are increased (Lönneborg *et al.*, 1985). The increase of PC is biphasic; rapidly in the first 10-15 h and then a slow increase is detected (Lönneborg *et al.*, 1985). However, the amounts of PBS decrease with no alteration in PBS composition or structure when *Microcystis aeruginosa* is shifted from low light to high light (Raps *et al* 1985). Complementary chromatic adaptation is clearly demonstrated when organisms are grown under red light and green light because PE and PC absorb different spectral qualities of light (Bogorad, 1975). Chromatic adapters are classified into three groups: Group I are those which cannot perform chromatic adaptation. Group II includes cyanobacteria which are able to synthesise PE alone to adapt the spectral quality of incident light. The synthesis of PE is enhanced in green light and repressed in red light, whereas the rate of PC synthesis is constant under both light regimes. Cyanobacteria able to synthesise both PC and PE and to adapt chromatically fall into Group III. *Synechocystis* sp. PCC 6803 belongs to Group I because it synthesises PC only and cannot perform complementary chromatic adaptation. *Fremyella diplosiphon* (also known as *Calothrix* sp. PCC 7601) is widely used for the studies of complementary chromatic adaptation (Bruns *et al.*, 1989; Oelmüller *et al.*, 1988a; Lomax *et al.*, 1987;

Conley *et al.*, 1988; Conley *et al.*, 1985; Oelmüller *et al.*, 1988b). When *Calothrix* sp. PCC 7601 are illuminated with green light, expression of *cpeA* and *cpeB* genes encoding α and β subunits of phycoerythrin (PE) are induced (Mazel *et al.*, 1986). Besides, two PE associated linker polypeptides, L_R^{35} and L_R^{36} (the linker polypeptides in the rod structures of the PBS with molecular weight of 35 and 36 kDa) are also found. None of the four proteins are detected when *Calothrix* sp. PCC 7601 is illuminated with red light. Mazel *et al.* (1986) also suggested that L_R^{35} and L_R^{36} are cotranscribed with the *cpeA* and *cpeB* genes. In *Calothrix* sp. PCC 7601, the RcaA protein, which is specific for the green-light signal, can bind to the *cpeBA* promoter (Sobczyk *et al.*, 1993). The RcaA protein is a phosphoprotein. Dephosphorylation of the RcaA protein prevents its binding to the *cpeBA* promoter region. Binding of RcaA to *cpeBA* promoter region needs a repeated hexanucleotides TTGTTA(N_4)TTGTTA (Sobczyk *et al.*, 1993). It is assumed that the repeated hexanucleotides are the DNA-binding site for RcaA because when the first TTGTTA is deleted RcaA is not able to bind to the DNA (Sobczyk *et al.*, 1993). There are two sets of genes encoding phycocyanin in *Calothrix* sp. PCC 7601. They encode constitutive and inducible phycocyanin (PC). In *Calothrix* sp. PCC 7601 the allophycocyanin (APC) gene and both PC gene sets are clustered in the genome (Conley *et al.*, 1986). The gene order is *apcA*, *apcB* (encoding α-APC and β-APC) *cpcB1*, *cpcA1* (which encode the constitutive β-PC, α-PC), and *cpcB2*, *cpcA2* (coding for inducible β-PC, α-PC)(Conley *et al.*, 1986). In *Calothrix* sp. PCC 7601, the RcaD protein, which is specific for the red-light signal, can bind to the *cpc2* (*cpcB2A2*) gene promoter (Sobczyk *et al.*, 1994). Like RcaA, RcaD is a phosphoprotein. Binding of RcaD to DNA also requires the TTGTTA(N_4)TTGTTA motif. When treated with alkaline phosphatase, the dephosphorylated RcaD protein cannot bind to the *cpc2* promoter region. Although both RcaA and RcaD need the TTGTTA(N4)TTGTTA motif, there are still two differences between them. Firstly, the repeated hexanucleotide motif is centred at -57 and -175 for RcaA and RcaD, respectively. DNase I digestion experiments showed that two regions

were protected by RcaD, while only one by RcaA (Sobczyk *et al.*, 1993 and 1994). Complementation of Fd33 (a red-light-indifferent mutant of *Calothrix* sp. PCC 7601) shows that the *rcaC* gene is necessary for Fd33 to restore the normal chromatic adaptation (Chiang *et al.*, 1992). The 71 kDa RcaC protein shows strong homology to some regulatory proteins in two-component regulatory systems involved in signal transduction (for review see Stock *et al.*, 1989; Albright *et al.*, 1989). RcaC exhibits a strong identity with PhoP in *Bacillus subtilis* (Seki *et al.*, 1987). However, it is not yet known whether or not RcaC is a phosphoprotein.

1.3.7. Phycobilisome and nutrient deprivation

There is evidence showing that phycobiliproteins serve as a nitrogen source in cyanobacteria when they suffer from nutrient starvation, especially nitrogen starvation (Allen and Smith, 1969; Boussiba and Richmond, 1980). During nutrient starvation, phycobiliproteins are broken down and the amino acids derived from the phycobiliproteins are used for *de novo* biosynthesis of proteins essential for cell division (Elmorjani and Herdman, 1987; Duke *et al.*, 1989). It is suggested that nitrogen starvation induces the synthesis of new proteases (Yamanaka and Glazer, 1980). Several lines of evidence have shown that protease activities are involved in the degradation of phycobiliproteins (Foulds and Carr, 1977; Boussiba and Richmond, 1980; Wood and Haselkorn 1980; Elmorjani and Herdman, 1987). It is also suggested that a metabolite of glucose will irreversibly inhibit the protease activity for PBS degradation in *Synechocystis* sp. PCC 6803 (Elmorjani and Herdman, 1987). Carbon starvation and chloramphenicol inhibit the protease activity induced by nitrogen starvation (Boussiba and Richmond, 1980). Degradation of phycobiliproteins in *Anabaena* ATCC 29413, *Anabaena* PCC 7118 and *Anabaena variabilis* met-38 (a methionine auxotroph of *Anabaena* ATCC 29413) does not involve guanosine tetraphosphate (Wood and

Haselkorn 1980), which increases proteolysis in *E. coli* (Cashel, 1969; St. John *et al.*, 1978). Also, starvation for methionine does not induce phycobiliprotein breakdown and protease activity (Wood and Haselkorn 1980). When *Synechococcus* sp. PCC 7942 is deprived of an essential nutrient, the PBS are specifically and rapidly degraded. Recently an *nblA* gene has been cloned and sequenced (Collier and Grossman, 1994). The NblA protein containing 59 amino acids, with a molecular mass of 7 kDa is essential for degradation of phycobilisomes in *Synechococcus* sp. PCC 7942. There is evidence showing that the NblA protein is a general effector of degradation of PBS in phosphorus- and nitrogen- or sulphur-deprived cells. Inactivation of *nblA* also demonstrates that it is not essential for growth. It is also suggested that NblA is directly responsible for PBS degradation or is the only component of the PBS-degrading machinery that is not essential for cell viability. Collier and Grossman (1994) suggested that NblA is the phycocyaninase found in *Anabaena cylindrica* (Foulds and Carr, 1977), or alternatively, that NblA directly alters the activity or specificity of a general protease.

1.4. State transitions

Both PS I and PS II absorb different spectral qualities of light. Light preferentially absorbed by PS I is referred to as PS I light; light preferentially absorbed by PS II is PS II light. When *Chlorella pyrenoidosa* cells are grown in the dark, followed by illumination under PS II light, the maximum state of oxygen evolution is called state 2 When *Chlorella pyrenoidosa* cells are illuminated under PS I light, the new maximum state of oxygen evolution is called state 1 (Fig. 1.8) (Bonaventura and Myers, 1969). Since both PS I and PS II absorb different spectral qualities of light and are linked together by an electron transport chain, equilibrium between the working rate of PS II and PS I is of great importance. State 1 to state 2 transitions (state transitions) are involved in maintenance of the equilibrium of the working rate of both photosystems. Several lines of

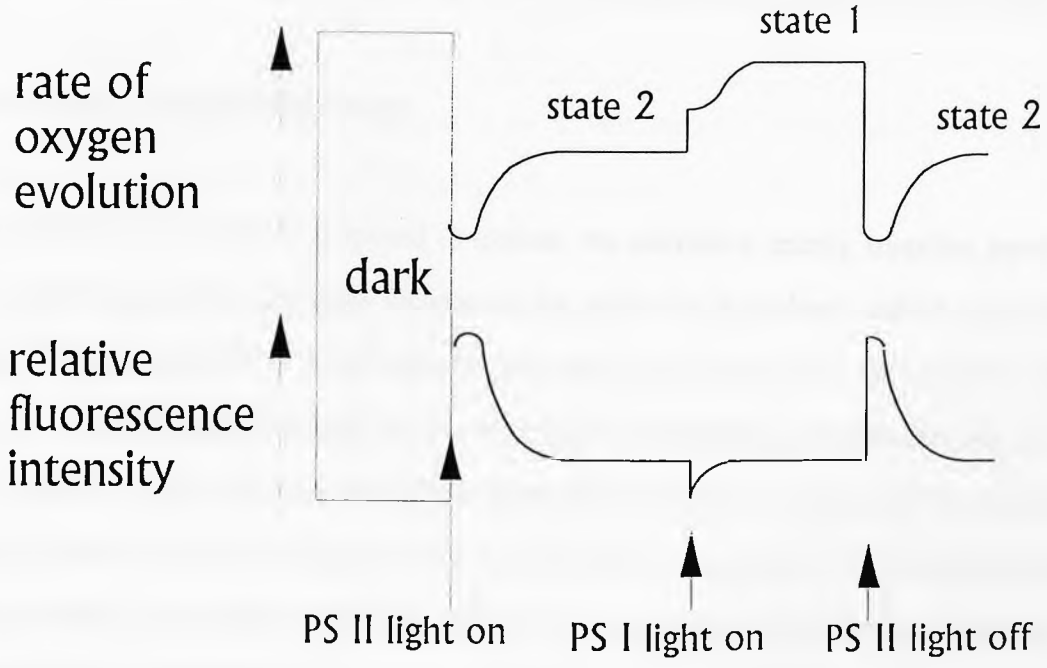

Fig. 1.8. Model State 1 to State 2 transitions (Redrawn from Bonaventura and Myers, 1969).

PS II light (λ=645 nm) and PS I light (λ=710 nm) were modulated. The oxygen and fluorescence signals were obtained through a lock-in amplifier. Fluorescence was measured at 686 nm. The maximum state of oxygen evolution and the redistribution of excitation energy were described in the text.

evidence have shown that state transitions are linked to phosphorylation and dephosphorylation of photosynthetic proteins by which the excitation energy is transferred between PS II and PS I (for review see Fork and Satoh 1986; Allen, 1992). State 2 leads to the phosphorylation of proteins and the excitation energy is consequently transferred to PS I from PS II. Under the illumination of PS I light, which results in state 1, protein phosphatase(s) is/are activated and phosphorylated proteins are dephosphorylated, thereby blocking the excitation energy transferred from PS II to PS I.

1.4.1 Transfer of excitation energy

Two hypotheses have been proposed to explain the excitation energy transfers between PS I and PS II. Early hypotheses focused on the spill-over hypothesis, which stated that phosphorylation of LHC II would transfer its energy to a closed PS II unit and then spill over to a neighbouring PS I unit. Studies of thylakoid membranes nevertheless show that PS II and PS I are not side-by-side in the thylakoid membranes. Instead, PS II occurs in stacked membranes while PS I is located in nonstacked membranes. The phosphorylated protein mobility hypothesis states that part of PS II can migrate from stacked membranes to non-stacked membranes as a result of induction of negative charges of phosphate group to protein molecules (for review see Bennett, 1991; Allen, 1992). The protein mobility hypothesis is strongly supported by evidence that migration of phosphorylated LHC II from PS II to PS I occurs (McCormac *et al.*, 1994). The comparisons of state 1 and state 2 are listed and summarised in table 1.3.

State 1 (illumination under PS I light)	State 2 Illumination under PS II light)
PQ pool is oxidised	PQ pool is reduced
Cyt *b6/f* is oxidised	Cyt *b6/f* is reduced
Phosphatase(s) is/are activated	Kinase(s) is/are activated
Protein dephosphorylation	Protein phosphorylation
Excitation energy transferred from PS II to PS I is blocked	Excitation energy transfers to PS I from PS II

Table. 1.3. The comparisons between state 1 and state 2. Abbreviations: PQ pool, plastoquinone pool; Cyt b6/f, cytochrome b6/f.

1.4.2 Excitation energy transfer in Cyanobacteria

Free bilin groups are cyclohelical shape, and have a strong UV-absorption band and a weaker absorption in the visible range. Bilin groups in a given phycobiliprotein have stronger visible absorption bands because they are held in extended conformations (for review see Glazer, 1989). Free bilin groups fluoresce very weakly; however, phycobiliproteins fluoresce strongly and the fluorescence comes from the bilin groups with the longest wavelength absorption bands.

The efficiency of transfer of light energy absorbed by phycoerythrin to phycocyanin is 99% and it takes only three steps to transfer the light energy absorbed from the distal rod phycoerythrin to the core of PBS (for review see Glazer, 1989). As described in section 1.3.2 the light energy absorbed by PE is sequentially transferred to PC, to APC and then to PS II reaction centres. However, in UV6P, an *apcA*-defective mutant of *Synechocystis* sp. PCC 6803, the assembly of rods is independent of the core and the light energy absorbed by rods can be directly transferred to PS I (Su *et al.*, 1992).

1.5. Protein phosphorylation and dephosphorylation

Reversible covalent modification of proteins plays important roles in the regulation of enzyme activities and protein functions. Many well-characterised post-translational modification events involve phosphorylation (for review see Cozzone, 1988; Bennett, 1991; Allen, 1992), alkylation (Golden and Bernlohr, 1989; Young and Bernlohr, 1990; Young and Bernlohr, 1991) and nucleotidylation (Foster et al., 1989; Silman *et al.*, 1995; for review see de Murcia *et al.*, 1995). However, only the phosphorylation of protein will be described in detail in this study.

Since protein phosphorylation was first detected in prokaryotic cells, it has been established to occur in more than 30 different prokaryotes including archaebacteria, as well as cyanobacteria (for review see Cozzone, 1988; Mann, 1995). Phosphorylated amino acid residues in proteins are classified into four main groups. (a) O-phosphomonoester linkages, such as serine, threonine and tyrosine. (b) N-phosphomonoester linkages, such as histidine. (c) Acyl phosphate, such as aspartic acid and glutamic acid (d) S-phosphomonoester linkages, such as cysteine. Hubbard and Cohen (1993) proposed a new mechanism for the regulation of phosphorylation and dephosphorylation of proteins, termed "the targeting subunit hypothesis" (Figure 1.9). This hypothesis states that protein kinases or protein phosphatases comprise a target subunit and a catalytic subunit. The target subunit will direct the catalytic subunit towards the target locus. Besides, the target subunit may allosterically alter the catalytic activity of the catalytic subunit. The target locus can be an organelle, a membrane or a component of the soluble fraction in the cell.

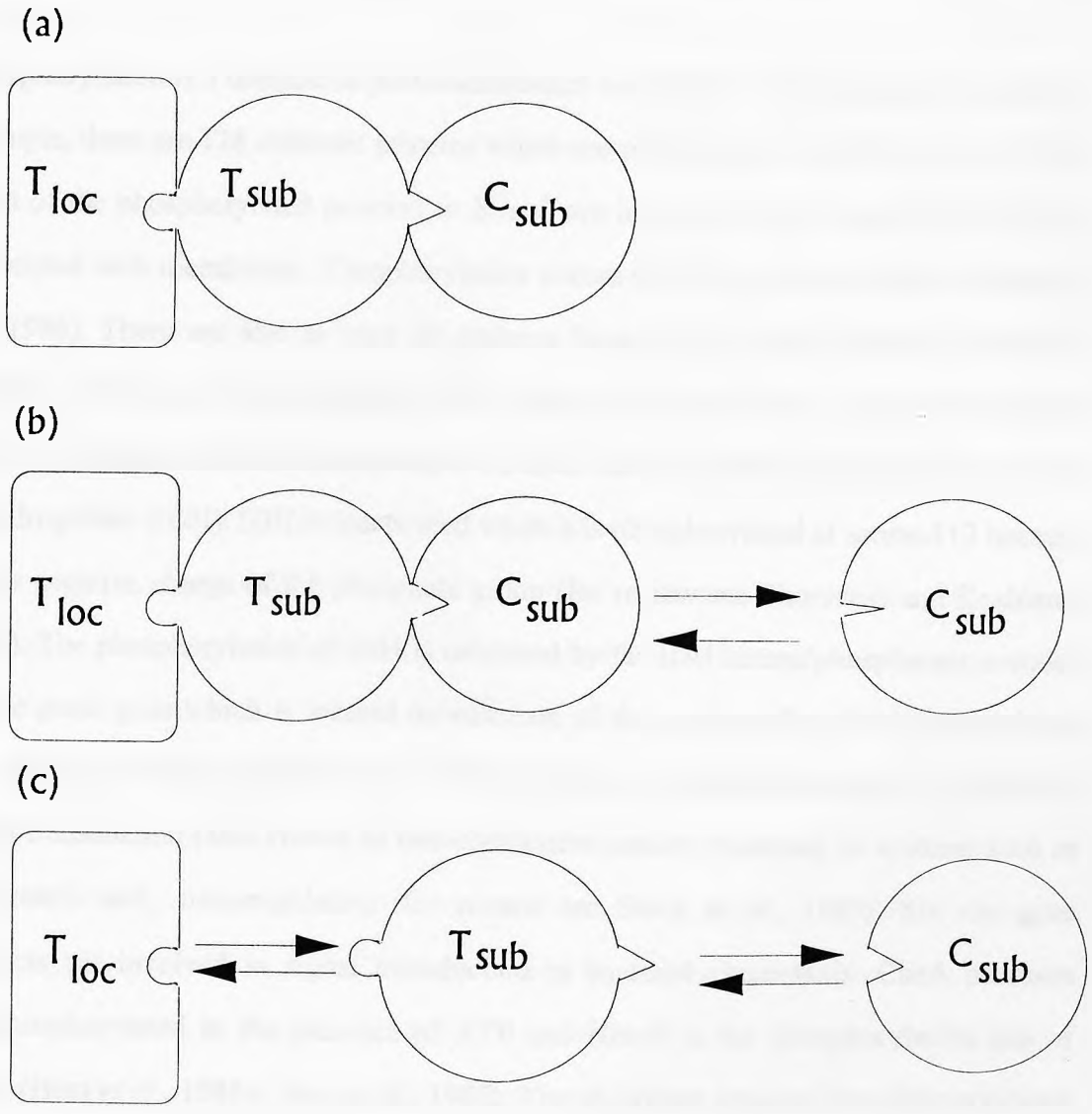

Fig. 1.9. Diagram of targeting subunit hypothesis (Adapted from Hubbard and Cohen, 1993). The targeting subunit (T_{sub}) is a part of a protein kinase or phosphatase which directs the catalytic subunit (C_{sub}) to the target locus (T_{loc}), as shown in (a). In (b), the interaction between a T_{sub} and a C_{sub} may also alter (allosterically) the catalytic activity or the specificity of the C_{sub} for nearby substrate. In (c), if the binding equilibrium between T_{sub} and either C_{sub} or T_{loc} is regulated, the altered properties of the C_{sub} can be switched on and off reversibly.

1.5.1. Protein phosphorylation in prokaryotes

Phosphorylation is a ubiquitous post-translational modification of proteins. In *E. coli*, for example, there are 128 different proteins which are phosphorylated (Cortay *et al.*, 1986). Most of the phosphorylated proteins in *E. coli* are located in cytosol and only three are associated with membranes. Phosphorylation occurs mostly at serine residues (Cortay *et al.*, 1986). There are also at least 30 proteins found to be phosphorylated in *Bacillus subtilis* (Köhler and Antranikian, 1989). Protein phosphorylation may affect enzyme activity. The most well-known example is, in *E. coli*, the phosphorylation of isocitrate dehydrogenase (IDH). IDH is inactivated when it is phosphorylated at serine-113 because of the negative charge of the phosphate group (for review see Thorsness and Koshland, 1987). The phosphorylation of IDH is catalysed by the IDH kinase/phosphatase encoded by the *aceK* gene which is located downstream of the genes coding for isocitrate lyase and malate synthase (Laporte *et al.*, 1985). Protein phosphorylation also involved in signal transduction (also known as two-component sensory systems), in systems such as chemotaxis and osmoregulation (for review see Stock *et al.*, 1989). Six *che* gene products are involved in signal transduction in bacterial chemotaxis. CheA becomes autophosphorylated in the presence of ATP and His-48 is the phosphorylation site of CheA (Hess *et al.*, 1988a; Hess *et al.*, 1987). The phosphate group of the phosphorylated CheA is transferred to CheB or CheY and then to some other *che* gene products (Hess *et al.*, 1988b). Hess *et al.*, (1988b) suggested that bacterial signal transduction is a flow of phosphate through a cascade of phosphorylated proteins. Protein phosphorylation is involved in nutrient regulation, such as the nitrogen utilisation genes, *glnALG* operon, and *glnB* and *glnD* genes, which have been widely investigated (for review see Magasanik, 1988). Protein phosphorylation is also found in photosynthetic bacteria as well. The α-subunit of the light-harvesting complexes I in *Rhodobacter capsulatus* (LHI) is phosphorylated by a membrane-bound protein kinase and the phosphorylation is under

redox control (Cortez *et al.*, 1992). *In vivo* labelling shows at least 25 proteins are phosphorylated in *Rhodomicrobium vannielii* and protein phosphorylation is dependent on the growth stage of cell culture (Turner and Mann, 1986). Two gene products, RegA and RegB, controlling anaerobic induction of light-harvesting complexes and reaction centre gene expression, are found to be phosphorylated at histidine residues *in vitro* in *Rhodobacter capsulatus* (Inoue *et al.*, 1995). Sequence and mutational analysis indicates that RegA and RegB resemble the components of a two component sensory system (Inoue *et al.*, 1995). Phosphorylation of light-harvesting complex B880 in *Rhodospirillum rubrum* has also been found and is under redox control (Holmes and Allen, 1988). An 88 kDa protein is phosphorylated at tyrosine residue in the photosynthetic bacterium *Prochlorothrix hollandica* (Warner and Bullerjahn, 1994). Phosphorylation of this 88 kDa protein is light-dependent, and dephosphorylation of this 88 kDa protein occurs in the dark. Protein phosphorylation also occurs in sugar phosphotransferase system (PTS) (for review see Stock, 1993; Saier *et al.*, 1995). Several lines of evidence also show that the PTS, like signal transduction, is also a flow of phosphate through a cascade of phosphoproteins; however, sugar is the final acceptor of the phosphate group (Peri *et al.*, 1984; for review Saier *et al.*, 1995). The PTS comprises three soluble proteins, enzyme I, histidine-containing protein (HPr), factor III and a membrane-bound protein, enzyme II. Enzyme I and HPr are two non-specific proteins in PTS, whereas factor III and enzyme II are specific for a certain sugar. Enzyme I is phosphorylated at a single histidine and the phosphate group comes from phosphoenolpyruvate (PEP) instead of ATP or the other nucleoside triphosphates. Phosphorylated enzyme I transfers the phosphate group to His-15 in HPr. Phosphorylated HPr will transfer its phosphate group to either factor III or to sugar through the membrane enzyme II. The phosphate group of the His-15 in HPr comes from PEP and/or enzyme I, but serine residue in HPr can also be phosphorylated by an ATP-dependent kinase (Deutscher and Saier, 1983; Reizer *et al.*, 1984). However, the seryl-bound phosphate

group cannot be transferred from HPr to sugar. Therefore, phosphorylation at a serine residue in HPr is believed to regulate sugar uptake (Deutscher and Saier, 1983). Phosphoserine in HPr is dephosphorylated by a soluble protein phosphatase, and a membrane-bound phosphatase is also found to hydrolyse this O-phosphomonoester linkage (Deutscher *et al.*, 1985). For controlling the cell mobility, serine residue of a 55 kDa protein in *Mycoplasma gallisepticum* is found to be phosphorylated (Platt *et al.*, 1988). The reversible phosphorylation of the 55 kDa protein could be involved in gliding mobility. Tyrosine is also found to be phosphorylated in the flagella from *Pseudomonas aeruginosa* PAO1 and M-2 strains (Kelly-Wintenberg *et al.*, 1990). Protein phosphorylation is also involved in cell differentiation. The *pkn1* gene of *Myxococcus xanthus* encodes a protein kinase related to eukaryotic protein kinases (Muñoz-Dorado *et al.*, 1991). This kinase autophosphorylates itself at both serine and threonine residues. The expression of this *pkn1* gene occurs immediately before spore formation. Deletion of the *pkn1* gene results in prematurity and poor spore formation (Muñoz-Dorado *et al.*, 1991). Protein phosphorylation is also found in the strictly anaerobic bacterium *Clostridium sphenoides* (Antranikian *et al.*, 1985a). In *C. sphenoides,* citrate lyase ligase is activated by phosphorylation, resulting in acetylation of citrate lyase which is the active form of the enzyme and degrades citric acid (Antranikian *et al.*, 1985b). Protein phosphorylation also plays important roles in transcription (Amster-Choder *et al.*, 1989; for review, see Magasanik, 1988; Collado-Vides *et al.*, 1991) and translation in *E. coli* (Lippmann *et al.*, 1993) and genetic competence in *Bacillus subtilis* (for review see Dubnau, 1991).

1.5.2. Protein phosphorylation in cyanobacteria

Protein phosphorylation has been reported in only four species of cyanobacteria, *Synechococcus* sp. PCC 6301 (Allen *et al.*, 1985), *Anabaena* sp. PCC 7120 (Mann *et al.*, 1991), *Synechocystis* sp. PCC 6803 (Bloye *et al.*, 1992) and *Calothrix* sp. PCC 7601 (Schuster *et al.*, 1984)

In cyanobacteria, protein phosphorylation is thought to be involved in state transitions, chromatic adaptation, inorganic nutrient uptake, stress responses and signal transduction (for review see Mann, 1995). In *Calothrix* sp. PCC 7601, the RcaA, RcaC and RcaD are all phosphorylated proteins which play important roles in chromatic adaptation (refer to Section 1.3.6). Photosynthetic protein is also phosphorylated, like those in green plants. A 6 kDa *psbH* gene product is phosphorylated both in the light and in the dark in *Synechocystis* sp. PCC 6803 (Race and Gounaris, 1993). However, phosphorylation of this *psbH* gene product is inhibited by DCMU and Zn^{2+}. Stress will also affect protein phosphorylation. In *Synechocystis* sp. PCC 6803, a 20.5 kDa protein is phosphorylated both *in vivo* and *in vitro* after salt shock (Hagemann *et al.*, 1993). In *Anabaena* sp. PCC 7120, the *pknA* gene product appears to be a eukaryotic serine/threonine type protein kinase (Zhang, 1993). It is suggested that PknA is required for the cell growth and differentiation (Zhang, 1993). The P_{II} protein, which is the *glnB* gene product, has been found to be uridylylated in *E. coli* (for review see Magasanik, 1988) and to be phosphorylated in cyanobacteria *Synechococcus* sp. PCC 6301 and PCC 7942 on a serine residue (Harrison *et al.*, 1990; Tsinoremas *et al.*, 1991, Forchhammer and Tandeau de Marsac., 1994). In *Synechococcus* sp. PCC 6301 phosphorylation of P_{II} is found to be dependent on the N-status and on state transitions (Tsinoremas *et al.*, 1991). However, phosphorylation of P_{II} protein in *Synechococcus* sp. PCC 7942 is independent of a specific light quality; interestingly, the dark shift experiment shows that P_{II} becomes dephosphorylated when nitrate is present (Forchhammer and Tandeau de Marsac., 1994).

P_{II} is also found to be a trimer in *Synechococcus* sp. PCC 7942 and different phosphorylated forms of P_{II} protein have also been found (Forchhammer and Tandeau de Marsac., 1994).

1.6. Protein kinases

Protein kinases, by definition, are enzymes that transfer a phosphate group from a phosphate donor to acceptors (amino acids) in substrate proteins. Generally, the phosphate group donor is the γ phosphate of ATP or another nucleoside triphosphate (for review see Hunter, 1991). Phosphoproteins may sometimes be a phosphate group donor as well. Most protein kinases need Mg^{2+} for their activities, but phosphorylation of some proteins may be inhibited in high concentration of Mg^{2+} (Mann *et al.*, 1991). On the basis of phosphorylation on different amino acid residues, protein kinases are classified into groups (for review see Hunter, 1991).

1.6.1. Classification of protein kinases

Since most protein kinases have multiple substrates, it would be sensible to classify protein kinases on the basis of the specific acceptor amino acid rather than specific protein substrate. The Nomenclature Committee of the International Union of Biochemists has recommended that protein kinases be classified as follows (for review see Hunter, 1991):

1. Phosphotransferases with a protein alcohol group as acceptor called protein-serine/threonine kinases (E.C. 2.7.10).
2. Phosphotransferases with a protein phenolic group as acceptor called protein-tyrosine kinases (E.C. 2.7.11).

3. Phosphotransferases with a protein histidine, arginine, or lysine group as acceptor called protein-histidine kinases (E.C. 2.7.12).
4. Phosphotransferases with a protein cysteine group as acceptor called protein-cysteine kinases (E.C. 2.7.13).
5. Phosphotransferases with a protein acyl group as acceptor called protein-aspartyl or glutamyl kinases (E.C. 2.7.14).

Enzymes of the first two categories are well known, whereas those in the last three categories are less well documented and characterised. Therefore, only the protein-serine/threonine and protein-tyrosine kinases will be discussed here.

1.6.2. Ser/Thr type protein kinase

Most of the protein kinases found are ser/thr type protein kinases, such as cAMP dependent protein kinase, protein kinase C, Ca/calmodulin dependent kinases, AMP activated kinase, casein kinases. More than 200 phosphorylation site sequences, in terms of protein ser/thr kinases, have been reported. The consensus specificity motifs are also tabulated (Pearson and Kemp, 1991). Protein kinases usually require substrate proteins to detect the kinase activities. Recently many kinds of synthetic peptides have been applied to the detection of protein kinase activities, especially ser/thr type protein kinases (for review see Pinna and Donella-Deana, 1994). Some synthetic tyrosyl peptides can also serve as substrates for tyr-specific protein kinases, but they are not as useful as those for ser/thr type protein kinases. Some protein kinase can autophosphorylate themselves on serine and/or threonine residues (Toner-Webb *et al.*, 1992, Sood *et al.*, 1994). In *E. coli* Era, an essential protein which binds both GTP and GDP has an intrinsic GTPase activity. The autophosphorylation reaction of Era is specific for GTP rather than ATP as a phosphoryl group donor. Phosphorylation of thr-36 or ser-37 is required for the function

of Era *in vivo*, which suggests that the Era autophosphorylation is likely play an important physiological role in *E. coli* (Sood *et al.*, 1994).

1.6.3. Protein-tyrosine kinase (PTK)

Protein tyrosine phosphorylation was regarded as an exclusively eukaryotic phenomenon. These enzymes play important roles in the control of the cell cycle (for review see Jacobs, 1992 and Wang, 1992). Over 40 phosphorylation site sequences of PTKs have been reported (for review see Pearson and Kemp, 1991). Recently an 88 kDa protein has been found phosphorylated on tyrosine residues under high light conditions in the prochlorophyte *Prochlorothrix hollandica* (Warner and Bullerjahn, 1994). The phosphorylated 88 kDa protein could be dephosphorylated under low light. This experiment also shows that protein tyrosine phosphorylation may be linked to the changes of the environmental conditions in prokaryotes.

1.7. Phosphatases

The Phosphate group of phosphoproteins can be removed by three kinds of enzymes, namely, phosphotransferases, phosphatases, and protein kinases which reverse the phosphorylation reaction. Both phosphotransferases and protein kinases need acceptors to receive the phosphate group. In this section, only phosphatases and some special kinases/phosphatases will be discussed. Not only can protein phosphatases remove the phosphate group from phosphoproteins, but many acid and alkaline phosphatases also express protein phosphatase activity at pH values close to neutrality (for review see Pinna and Donella-Deana, 1994). Little is known about protein phosphatases in prokaryotes. Ser/thr type protein phosphatase activity in the archaebacteria, for example, was first detected by Kennelly *et al.* (1993). Also, protein tyrosine phosphatase activity was first identified in a prokaryote by Potts (1993). Protein phosphatase and protein kinase activities may also be located in different domains of a same protein molecule. In *E. coli* ML308, isocitrate dehydrogenase (IDH) kinase/phosphatase is a well-known example (Nimmo *et al.*, 1984). Both the IDH kinase and phosphatase activities are completely lost when the kinase and phosphatase domains are separated. Additionally, the IDH phosphatase needs either ATP or ADP for its activity. In the presence of ADP, or ATP and an IDH kinase inhibitor, the IDH kinase/phosphatase can express its full phosphatase activity.

1.7.1. Classification of protein phosphatases

Most known protein phosphatases are ser/thr type phosphatases. The number of protein tyrosine phosphatases found is increasing. In addition, some acid and alkaline phosphatases also possess protein phosphatase activities.

1.7.2. Ser/Thr type protein phosphatases

Ser/thr type protein phosphatases can be classified into two main groups based on protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2). PP1 can dephosphorylate the β-subunit of phosphorylase kinase and is inhibited by two small heat- and acid-stable proteins, called inhibitor 1 (I-1) and inhibitor 2 (I-2). PP2 dephosphorylates the α-subunit of phosphorylase kinase and is insensitive to I-1 and I-2. PP2 can, in turn, be subclassified into three distinct enzymes, PP2A, 2B and 2C. PP2A, like PP1, can display its activity in the absence of divalent cations. PP2B and PP2C need Ca^{2+} and Mg^{2+} for their activities, respectively (for review see Cohen, 1989). In addition to Mg^{2+} and Ca^{2+}, some other divalent cations will also affect the activity of ser/thr protein phosphatase. Mn^{2+}, Ni^{2+}, Co^{2+} will stimulate the activity of the protein phosphatase found in archaebacterium *Sulfolobus solfataricus*, while EDTA, Cu^{2+}, Zn^{2+} will inhibit the phosphatase activity (Kennelly, 1993).

1.7.3. Protein tyrosine phosphatase (PTP)

Protein-tyrosine phosphorylation used to be regarded as exclusively occurring in eukaryotic cells. Potts and colleagues (1993) identified a gene product, IphP, from the chromosome of the cyanobacterium *Nostoc commune* UTEX 584 that contained the His-Cys-Xaa-Ala-Gly-Xaa-Xaa-Arg sequence which is characteristic of known protein tyrosine phosphatases. Many compounds modulate the activity of PTPs. Orthovanadate inhibits the activity of all PTPs tested so far. Molybdate, EDTA, Mn^{2+} and Zn^{2+} show different inhibitory effects on PTP activities. Besides, N-ethylmaleimide, p-(hydroxymercuri)benzoate and iodoacetate are irreversible inhibitors of PTPs (Pot and Dixon, 1992 and Fischer *et al.*, 1991). Comparison of amino acid sequences of protein tyrosine phosphatases shows that all these phosphatases, without exception, contain an

active site signature motif (I/V)HCXAGXGR(S/T)G (Guan and Dixon, 1987; Stone and Dixon, 1994).

1.7.4. Non-specific phosphatases

Some acid and alkaline phosphatase can dephosphorylate phosphoproteins. Thylakoid acid and alkaline phosphatase activities were reported in wheat (Yang *et al.*, 1987) and in pea thylakoid membranes (Michel *et al.*, 1987), respectively. This type of phosphatases hydrolyses the phosphate ester bond through a phosphoserine intermediate in the case of alkaline phosphatases, and through a phosphohistidine intermediate in the case of acid phosphatases (Stone and Dixon, 1994; Shinozaki *et al.,* 1995).

1.8. Photosynthesis and protein phosphorylation

Several kinds of protein kinases in eukaryotic cells have been found in the past years. As for photosynthesis, phosphorylation of thylakoid membrane proteins was first found by Bennett (1977) in pea thylakoids. More than one protein kinase which phosphorylates thylakoid membrane proteins in green plants has been reported (Bennett, 1979; Michel *et al.*, 1987 and Allen, 1992). In the cyanobacterium *Synechococcus* sp. PCC 6301 an 18.5 kDa protein which is a component of phycobilisome can be phosphorylated *in vivo* in state II, but not in state I (Sanders and Allen, 1987). The 18.5 kDa protein has been tentatively identified as β-phycocyanin (Harrison, 1990). Two protein kinases have been found in *Synechocystis* sp. PCC 6803 in this laboratory, one of which is light-dependent and the other is light-independent (N. J. Silman, personal communication). In *Synechocystis* sp. PCC 6803 several proteins can be phosphorylated *in vitro* in state I, while in state II a single 18 kDa protein can be phosphorylated. The properties of both kinases are described in Table 1.4. (Silman *et al.*, unpublished data).

light-independent kinase (LIK)		light-dependent kinase (LDK)	
active	inactive	active	inactive
PS I light			PS I light
dark			dark
	PS II light	PS II light	
DTT			DTT
	Ferricyanide	Ferricyanide	
Glucose-6-P			Glucose-6-P
	Ribulose-5-P	Ribulose-5-P	
	$NAD^+/NADP^+$	$NAD^+/NADP^+$	
NADH/NADPH			NADH/NADPH
	FSBA	FSBA	
DCMU		DCMU	

Table 1.4. The properties of Light-dependent kinase (LDK) and light-independent kinase (LIK) from *Synechocystis* sp. PCC6803 (Silman *et al.*, unpublished data). Illumination under PS I light and incubation in the dark and some kinds of chemicals, such as DTT (dithiothreitol), glucose 6-phosphate, NADH and NADPH will activate LIK and inactivate LDK. Illumination under PS II light, potassium ferricyanide, NAD+, NADP+, and FSBA (5'-*p*-flourosulfonylbenzoyladenosine) are activators and inactivators of LDK and LIK, respectively. DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) will inhibit both LDK and LIK.

In vivo labelling peas using [^{32}P] orthophosphate, Bennett (1980) also found that pea thylakoid membrane possessed protein phosphatase activity. In Bennett's experiment, a 26 kDa protein, which was derived from LHC II could be phosphorylated in the light and the phosphorylated 26 kDa protein can then be dephosphorylated in the dark. Dephosphorylation of the 26 kDa protein doesn't need Mg^{2+} for the phosphatase activity whereas dephosphorylation of a 9 kDa protein only takes place in the presence of Mg^{2+}. The protein phosphatase activity is destroyed by addition of the non-ionic detergent Triton X-100. Besides, the protein phosphatase activity is inhibited by NaF, a very common protein phosphatase inhibitor. Dephosphorylation of thylakoid membrane proteins is also not under the redox control (Bennett, 1980). Dephosphorylation of wheat thylakoid phosphoproteins is likely to be performed by the thylakoid protein phosphatase and thylakoid acid phosphatase, but the acid phosphatase plays a minor role (Yang *et al.*, 1987). As we know now, the thylakoid membranes possess not only protein kinase and protein phosphatase, but also acid and alkaline phosphatase. Some pea thylakoid phosphoproteins (45-, 30-, 26-and 9-kDa proteins) can be dephosphorylated by thylakoid protein phosphatase (Bennett, 1980), but other LHC II phosphoproteins (25- and 27-kDa proteins) are dephosphorylated by the thylakoid alkaline phosphatase (Michel *et al.*, 1987). Dephosphorylation of the 25- and 27-kDa proteins is performed by a membrane-bound alkaline phosphatase of optimum pH 9.0 and the activity of alkaline phosphatase has a requirement for Mg^{2+}. Unfortunately, no phosphatase linked to dephosphorylation of photosynthetic proteins in prokaryotes has been purified so far.

1.9. Aims of the project

Reversible covalent modification of proteins plays important roles in the regulation of enzyme activities and protein functions. Protein phosphorylation have been reported in green plants (for review see Allen, 1992) and in cyanobacteria (for review see Mann, 1995).

That thylakoid membranes possess protein kinase activities have been demonstrated in green plants (Bennett, 1977; Bennett, 1979a; for review see Bennett, 1991) and their role in state-transitions has been widely accepted (for review see Allen, 1992). Like green plants, cyanobacteria have also shown to exhibit state-transitions with concomitant phosphorylation of several thylakoid proteins (Sanders and Allen, 1987; Harrison *et al.*, 1991; Race and Gounaris, 1993).

Consequently, the overall aim of this project was to establish some aspects of the physiological roles of protein phosphorylation in cyanobacteria, especially focusing on the relationship between protein phosphorylation and photosynthesis.

Chapter 2

Materials and Methods

2.1. Biological materials

The cyanobacterial strain used in this study was *Synechocystis* sp. PCC 6803.

2.2. Non-biological materials

All chemicals used were of the highest grade commercially available. The suppliers of specialist materials, such as radiochemicals, are noted where appropriate. Solutions were made up in distilled water or high quality deionised water, referred to as Elga water.

2.2.1. Growth media

All growth media were made up in Elga water unless otherwise stated. All media were autoclaved at 121°C, 15 psi for 15 minutes. Non-autoclavable components were filtered through Acrodisc PF (0.2 μm) disposable filter units.

The composition of BG11 medium for the growth of *Synechocystis* sp. PCC 6803 is shown in Table 2.1

Table 2.1 Compositions of BG 11 medium. Adapted from Rippka *et al* 1979:

Constituents	$g.l^{-1}$
$NaNO_3$	1.5
$K_2HPO_4.3H_2O$	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Citric acid	0.036
$FeNH_4$ citrate	0.006
EDTA disodium salt	0.001
Na_2CO_3	0.02
Trace elements	1 ml

pH is adjusted to 7.4 using 1M HCl or 10 N KOH.

Trace elements	
Constituents	$g.l^{-1}$
H_3BO_3	0.572
$MnCl_2.4H_2O$	0.362
$ZnSO_4.7H_2O$	0.044
$NaMoO_4.2H_2O$	0.078
$CuSO_4.5H_2O$	0.016
$Co(NO_3)_2.6H_2O$	0.010

2.2.2. Commonly used buffers

(A) TES buffer pH 7.5 (TES 7.5) series:

TES 20 mM and TES 12 mM.

TES 24 mM containing 24 mM NaCl and 12 mM EDTA.

TES 12 mM containing 12 mM NaCl and 6 mM EDTA.

The pH of the buffer series was adjusted to 7.5 using 10 N NaOH.

(B) Phosphate buffer (PB 8.0):

0.75 M NaH_2PO_4 and 0.75 M K_2HPO_4 were made individually. An adequate volume of 0.75 M NaH_2PO_4 was added to 1 litre of 0.75 M K_2HPO_4 to make 0.75 M phosphate buffer, pH 8.0.

(C) MES buffer series:

MES buffer A: 20 mM MES containing 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 25% (final volume) of glycerol was made, and the pH was adjusted to 6.35 using 10 N NaOH.

MES buffer B: 20 mM MES containing 20 mM $CaCl_2$, 5 mM $MgCl_2$ and 25% (final volume) of glycerol was made, and the pH was adjusted to 6.35 using 10 N NaOH.

2.3. Culture of *Synechocystis* sp. PCC 6803

Cells of *Synechocystis* sp. PCC 6803 were routinely cultured in small flasks in the light (300 $\mu E.m^{-1}.s^{-1}$) at 30°C. Cells were transferred to new medium every week. Cells were cultured in a 12 litre fermenter (Duran, Mainz, Germany) in the light (300 $\mu E.m^{-1}.s^{-1}$) at 30°C.

2.3.1. Cell counting

Cell numbers were estimated using an AO counting chamber

2.3.2. Assay of chlorophyll content

Aliquots (20 µl) of *Synechocystis* sp. PCC 6803 culture were added to 10 ml of 80% acetone followed by vortexing. Protein was removed by centrifugation. The supernatant was collected and the absorbance at 663 nm was read. The concentration of chlorophyll was calculated on the basis of E_{663}=82.04 ml/mg ·cm. (Bendall *et al.*, 1988).

2.3.3. Assay of phycobiliprotein content

Cultures of *Synechocystis* sp. PCC 6803 cells (100 ml) were centrifuged at 10,000 x g for 10 min at 4°C. The cell pellets were washed in 20 mM sodium acetate buffer, pH 5.5, resuspended and broken by passage through a French pressure cell (section 2.4). Membranes containing chlorophyll were precipitated by addition of streptomycin sulphate (1% (w/v)) to the cell-free extracts, and left for 30 min at 4°C. The precipitate was removed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant containing phycobiliproteins was collected and absorbances at 565, 620 and 650 nm were measured. The amounts of different phycobiliproteins were calculated as follows (Tandeau de Marsac and Houmard, 1988):

$PC\ (mg.ml^{-1}) = (OD_{620\ nm} - 0.7 \times OD_{650\ nm}) \div 7.38$

$APC\ (mg.ml^{-1}) = (OD_{650\ nm} - 0.19 \times OD_{620\ nm}) \div 5.65$

$PE\ (mg.ml^{-1}) = (OD_{565\ nm} - 2.8[PC] - 1.34[APC]) \div 12.7$

2.3.4. Assay of protein concentration

Assay of protein concentration was performed using Bio-Rad protein assay solution. Aliquots of protein solution (800 μl) were added to Bio-Rad protein assay solution (200 μl) and mixed completely. Absorbance at 595 nm was read. Different concentrations of bovine serum albumin were also prepared to make standard curve.

2.3.5. Assay of protein kinase activity

Aliquots (36 μl) of either the thylakoid membranes or the blue fractions from a sucrose gradient (corresponding to phycobiliproteins) were incubated at 30°C for 10 min in the presence of $MgCl_2$/ATP (2 μl 100 μM $MgCl_2$; 2μl 10μM ATP) and [γ-^{32}P]ATP (5μ*Ci*/μM, 1μl). The reaction was stopped by adding sample buffer (13.3 μl) to the reaction mixtures (40 μl, total volume) and then heating at 100°C for 5 min. Phosphorylated protein bands were detected by means of polyacrylamide gel electrophoresis, followed by autoradiography.

2.3.6. Pulse-chase reaction

Aliquots of the thylakoid membranes or the phycobiliprotein-containing fractions of a sucrose gradient (36 μl) were incubated at 30°C for 5 and 10 min in the presence of $MgCl_2$/ATP (2 μl 100 μM $MgCl_2$; 2μl 10μM ATP) and [γ-^{32}P]ATP (5μ*Ci*/μM, 1μl). Aliquots (4μl) of ATP (10 mM) were added and further incubated for 5, 10 and 20 min. The reaction was stopped by adding sample buffer (13.3 μl) to the reaction mixtures and then heating at 100°C for 5 min. Phosphorylated protein bands were detected by means of electrophoresis, followed by autoradiography.

2.4. Preparation of the thylakoid membranes

Cultures of *Synechocystis* sp. PCC 6803 cells (1 litre) were centrifuged at 14,300 x g (Beckman JA10 rotor) for 10 min at room temperature. The cell pellets were washed twice in 20 mM TES buffer pH 7.5, resuspended and centrifuged at 13,800 x g (Beckman JA17 rotor) for 10 min at room temperature. Cells were resuspended in 15 ml 10 mM TES buffer pH 7.5 containing 2 mM EDTA, 0.2% lysozyme and 600 mM sucrose and were incubated at 30°C for 2 hr. Cells were collected at 7,000 x g (Beckman JA17 rotor) for 10 min at room temperature. The pellets were washed twice in 20 mM TES buffer pH 7.5 and were repelleted again. The cell pellets were resuspended in adequate amounts of 20 mM TES buffer pH 7.5 and broken by using French pressure cell (16,000 psi) at 4°C. Four passages were normally used for a better breakage of the cells (more than 90% cells were broken). Unbroken cells and cell debris were removed by centrifugation at 6,000 x g (Beckman JA17 rotor) for 10 min at 4°C. The supernatant was collected and the total membranes were obtained by centrifugation at 200,000 x g for 1 hr at 4°C. The total membranes were resuspended in 20 mM TES buffer pH 7.5 and layered on top of a sucrose density gradient and centrifuged at 130,000 x g (Beckman SW28 rotor) for 18 hr at 4°C. The preparation and volume of the sucrose density gradient were as follows:

90 g of sucrose were dissolved in 43.1 ml of 24 mM TES buffer containing 12 mM EDTA and 24 mM NaCl pH 7.5 to make 90 % sucrose buffer solution. 90 % sucrose buffer solution was diluted using 12 mM TES buffer containing 6 mM EDTA and 12 mM NaCl, pH 7.5 to make different concentrations of sucrose buffer solutions used for the preparation of the sucrose density gradient.

Conc. (%) and vol. (ml) of sucrose density gradient	90% sucrose (ml)	12 mM TES (ml)
10 %, 3 ml	1.11	8.89
30 %, 5 ml	3.33	6.67
45 %, 5 ml	5	5
50 %, 5 ml	5.55	4.45
60 %, 3 ml	6.66	3.34
70 %, 3 ml	7.77	2.23
80 %, 3 ml	8.88	1.12
90 %, 3 ml	3	0

2.5. Isolation of phycobilisomes (PBS)

2.5.1. Preparation of PBS

Synechocystis sp. PCC 6803 cells were harvested and washed twice in 0.75 M NaK_2PO_4 buffer containing 1 mM sodium azide, 1 mM β-mercaptoethanol, pH 8.0. Cells were resuspended in the same buffer supplemented with 1 mM EDTA and 1 mM benzamidine and were broken using a French pressure cell (16,000 Psi). Extracts were centrifuged at 17,000 x g (Beckman SW41 rotor) for 20 min. The pellets were treated with 2% Triton and 1 mM phenylmethylsulfonyl-fluoride (PMSF) for 30 min with gentle shaking in the dark. The membranes were removed by centrifuge at 17,000 x g (Beckman SW41 rotor) for 20 min. The supernatants were layered on top of a sucrose density gradients in 0.75 mM NaK_2PO_4 buffer, pH 8.0 and centrifuged at 98,000 x g (Beckman SW41 rotor) for 16 hr. The sucrose density gradients consisted of, from the bottom to the top, 1 ml of 2M, 2.5 ml of 1 M, 3.5 ml of 0.75 M, 2 ml of 0.5 M, and 1 ml of 0.25 M sucrose. PBS were

recovered in the lower part of the 0.75 M sucrose band (Elmorjani *et al.*, 1986). PBS are sensitive to the cold and the light and are stable in a high concentrations of phosphate buffer; therefore, all the steps were carried out at 20°C and avoided light (for review see Glazer, 1988a; Katoh, 1988).

2.5.2. Preparation of C-phycocyanin

Synechocystis sp. PCC 6803 cells were suspended in 50 mM ammonium acetate buffer, pH 6.0 and broken by passage through a French pressure cell (16,000 psi). Unbroken cells and cell debris were removed by centrifugation at 23,500 x g for 30 min at 4°C. The supernatant was collected, and the pellets were washed, centrifuged and the supernatant was collected again. Solid ammonium sulphate was added to the supernatant to make 65% saturation and allowed to stand for overnight at 4°C. Care was taken that the pH should not change when ammonium sulphate was added to an unbuffered or weakly buffered solution. The precipitate was collected by centrifugation at 27,000 x g for 15 min at 4°C and resuspended in 5 mM potassium phosphate buffer, pH 7.0 and dialysed against the same buffer for overnight at 4°C. The dialysed protein solution was centrifuged at 12,000 x g for 10 min at 4°C to remove denatured protein and membranes and subjected to anion exchange chromatography on an FPLC Hiload 26/10 Q Sepharose column (Pharmacia, Uppsala, Sweden). The proteins were eluted from the column using a linear 5-200 mM potassium phosphate buffer pH 7.0 gradient. All the fractions were collected and allowed to stand overnight. The absorbance at 615 nm and 650 nm of each fraction was measured.

2.6. Preparation of PS II particles

Synechocystis sp. PCC 6803 cells were pelleted and washed twice in MES buffer A (20 mM MES-NaOH (pH 6.35); 5 mM $CaCl_2$; 5 mM $MgCl_2$/ 25% glycerol (v/v)). Pellets were resuspended in buffer A containing 1 mM benzamidine and 1 mM PMSF. Cell suspensions were added into beadbeater chamber in which 0.1 mm glass beads were filled up to half of the height of the chamber. Cells were broken by 10 cycles of vibration (20 seconds in every 2 minutes) at 4°C, in the dark. The glass chambers were left for several minutes and the suspensions were collected. The glass beads were washed 4 to 5 times in buffer A. The washed buffer was collected together with the cell suspensions, followed by centrifugation at 2,000 x g for 5 min to remove the unbroken cells and glass beads. Membrane fragments were pelleted at 179,000 x g for 20 min (Beckman SW41 rotor). Pelleted membranes were suspended in MES buffer B (20 mM MES-NaOH pH 6.35; 20 mM $CaCl_2$; 5 mM $MgCl_2$/ 25% glycerol (v/v)). The membrane suspension was diluted to 1.2-1.5 mg chlorophyll/ml (section 2.3.2.). Membranes were solubilised with n-dodecyl β -maltoside (DM/chlorophyll = 1.25/1 (w/w)) and gently mixed in the dark at 4°C for 30 min. n-octyl β-glucopyranoside (OG/chlorophyll = 5/1 (w/w)) was added to the sample and gently mixed in the dark at 4°C for 30 min. The mixtures were then centrifuged at 164,000 x g (Beckman SW 41 rotor) at 4°C for 50 min. The supernatant enriched in PS II particles was carefully removed, followed by precipitation by adding 2 ml of 25% PEG 6000. Pellets were collected after centrifugation at 171,000 x g (Beckman SW41 rotor) at 4°C for 20 min. The pellets were resuspended in a minimal volume of MES buffer B and immediately used or frozen at -20°C (Kirilovsky *et al.*, 1992).

2.7. Isolation of chromosomal DNA from *Synechocystis* sp. PCC 6803

Cultures of *Synechocystis* sp. PCC 6803 cells grown to late log phase were collected by centrifugation at 6,000 x g (Beckman JA17 rotor) for 10 min at room temperature. The cells were resuspended in 0.5 ml of 0.25 M Tris-HCl buffer pH 8.0 containing 20% sucrose and 0.1% lysozyme followed by incubation at 37°C for 1 hr. 16 µl Sarkosyl (30%) and protease K (5 mg/ml, 20 µl) were added in the cell suspension and incubated at 65°C for overnight. An equal volume of phenol-chloroform was added to the cell suspension and mixed by vortexing. The suspensions were centrifuged in an Eppendorf tube for 4 min. The upper layer containing chromosomal DNA was removed to another Eppendorf tube. Chloroform/isoamyl alcohol (24:1) mixture was added to the chromosomal DNA solution and mixed completely. The upper layer was removed to an Eppendorf tube after centrifugation for 4 min. 2 volumes of isopropanol and 0.4 volume of 7.5 M ammonium acetate were added to the supernatant. The DNA mixtures were chilled at -20°C for at least 1 hr (overnight was preferred), followed by centrifugation for 10 min. The DNA pellets were washed twice in 70% ethanol and repelleted by centrifugation. Ethanol was discarded and the chromosomal DNA pellets were dried in a desiccator under vacuum. The dried DNA pellets were dissolved in an adequate volume of sterile H_2O and stored at -20°C (Porter, 1988).

2.7.1. Restriction digestion of chromosomal DNA from *Synechocystis* sp. PCC 6803

Aliquots of *Synechocystis* sp. PCC 6803 chromosomal DNA (5 µl) were added to an Eppendorf tube in which mixtures of enzyme buffer containing spermidine (40 mM, 2µl), a suitable restriction enzyme buffer (5 units), H_2O (11µl) were added, and were mixed thoroughly. A suitable restriction enzyme was added to the mixture, followed by

incubation at 37°C for 2 hr. The restriction digested *Synechocystis* sp. PCC 6803 chromosomal DNA was used immediately or frozen at -20°C.

2.8. Electrophoresis of proteins — SDS PAGE

2.8.1. Solutions

(A) Acrylamide Mix:

Acrylamide	60.0 g
Bis-acrylamide	1.6 g

H_2O was added to a final volume of 200 ml.

(B) Resolving Gel Buffer:

Tris base (Trizma base)	36.3 g
1M HCl	48.0 ml

H_2O was added to a final volume of 100 ml and the pH was adjusted to 8.8.

(C) Stacking Gel Buffer:

Tris base (Trizma base)	6.0 g
1M HCl	40.0 ml

H_2O was added to a final volume of 100 ml and

the pH was adjusted to 6.8.

The acrylamide solution, resolving gel buffer and stacking gel buffer were filtered through Whatman No. 1 filter paper.

(D) Running Buffer:

Glycine	8.64 g
Tris base (Trizma base)	1.8 g
SDS	o.6 g

H_2O was added to a final volume of 600 ml.

(E) Ammonium persulphate (APS):

75 mg of APS was dissolved in 5 ml of H_2O to make 1.5 % APS. APS was freshly made.

(F) Sample buffer:

Stacking gel buffer, solution (B)	2.5 ml
SDS (10%)	4.0 ml
β-mercaptoethanol	2.0 ml
Glycerol	2.0 ml

If necessary, tracking dye (bromophenol blue) was added to the sample buffer.

2.8.2. Compositions of different concentrations of polyacrylamide

(ml)	stacking gel	Resolving gel							
		20.0%	17.5%	15.0%	12.5%	11.0%	10.0%	7.5%	5.0%
Solution (A)	2.5	20.0	17.5	15.0	12.5	11.0	10.0	7.5	5.0
Solution (B)		3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Solution (C)	5.0								
10 % SDS	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
1.5 % APS	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
H_2O	11.3	4.45	6.95	9.45	11.95	13.45	14.45	16.95	19.45
TEMED	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015

2.9. Electrophoresis of proteins — SDS Gradient PAGE

2.9.1. Solutions

(A) Acrylamide Mix (low Bis):

Acrylamide	60.0 g
Bis-acrylamide	0.3 g

H_2O was added to a final volume of 100 ml.

(B) Acrylamide Mix (high Bis):

Acrylamide	60.0 g
Bis-acrylamide	1.6 g

H_2O was added to a final volume of 100 ml.

(C) Stacking gel solution:

Acrylamide	10.0 g
Bis-acrylamide	0.5 g

H_2O was added to a final volume of 100 ml.

(D) Resolving Gel Buffer and (E) Stacking Gel Buffer are described previously (section 2.8.1.).

(F) Running buffer:

(1) for SDS Disc gradient gel

Tris base (Trizma base)	1.8 g
Glycine	8.64 g
SDS	0.6 g

H_2O was added to a final volume of 600 ml

(2) for Native Disc gradient gel

Tris base (Trizma base)	12.0 g
Glycine	57.6 g

H_2O was added to a final volume of 2 litre

2.9.2 Compositions of 30%-10% SDS polyacrylamide solution

	resolving gel		stacking gel
(ml)	30% Polyacrylamide	10% Polyacrylamide	
solution (A)	10		
solution (B)		8.3	
solution (C)			3.0
75 % Glycerol	7.3		
H_2O		34.9	4.4
solution (D)	2.5	6.25	
solution (E)			2.4
SDS (10 %)	0.2	0.5	0.1
TEMED	0.004	0.01	0.005
APS (10 %)	0.04	0.1	0.01

2.10. Electrophoresis of protein — Native gradient PAGE

2.10.1. Solutions

With the exception of solution (C), solution (E), and 10 % SDS, all the solutions used for the preparation of native disc gradient PAGE are the same as those used for SDS disc gradient PAGE.

2.10.2. Compositions of 4-30% native polyacrylamide solution

(ml)	30% polyacrylamide	4% polyacrylamide*
solution (A)	10	
solution (B)		3.32
75 % Glycerol	7.4	
H_2O		40.38
solution (D)		6.25
TEMED	0.01	0.02
APS (10 %)	0.1	0.2

* 5 ml from the 4 % polyacrylamide solution were taken out to make "tooth former" before the TEMED and APS were added. 10µl of TEMED and 0.1 ml of 10% APS were added to the "tooth former" solution and subsequently layered on top of the gel.

2.11. Casting linear and gradient PAGE gels

2.11.1. Linear PAGE gels

The resolving gel mixture was degassed. Immediately before pouring adequate TEMED was added. After casting, the gel was overlayered with water-saturated butan-2-ol and allowed to set. When the gel had set, the water-saturated butan-2-ol was washed off. TEMED was added to the degassed stacking gel followed by pouring the stacking gel onto the top of the gel and an appropriate comb was inserted.

2.11.2. Gradient PAGE gels

Silicon rubber tubing which passed through a peristaltic pump was attached to a 21Gx1.5 needle (Sabre, Sabre International Products Ltd.) which was used to pierce a size 41 Suba seal. A piece of plastic tubing (1 mm internal diameter) was forced through the Suba seal. The tubing was long enough to reach the bottom of a 20 ml glass universal bottle. The other end of the tubing was taped to the assembled casting plates. Both high and low concentration gels were degassed, followed by the addition of TEMED. The low concentration gel was pumped through the 21G needle. The Suba seal was inserted into the universal bottle containing the high concentration gel and a magnetic stirring bar. The peristaltic pump was then started to deliver the low concentration gel to the universal bottle at full speed and mixed with the high concentration gel. The gel was then overlaid with water-saturated butan-2-ol and allowed to polymerise for at least 3 hours. When the gel had set, the butan-2-ol was washed off. TEMED was added to the stacking gel or tooth former followed by pouring it on the top of gel and an appropriate comb was inserted.

2.12. Tricine gel electrophoresis

2.12.1. Solutions

(A) Acrylamide A solution:

Acrylamide	48 g
Bis-acrylamide	1.5 g

H_2O was added to a final volume of 100 ml.

(B) Acrylamide B solution:

	46.5 g
Acrylamide	
Bis-acrylamide	3.0 g

H_2O was added to a final volume of 100 ml.

(C) Gel buffer:

Tris base (Trizma base)	36.3 g
SDS	3.0 g

H_2O was added to a final volume of 100 ml and the pH was adjusted to 8.45.

(D) Bottom tank buffer:

Tris base (Trizma base)	7.26 g

H_2O was added to a final volume of 300 ml and the pH was adjusted to 8.9.

(E) Top tank buffer:

Tris base (Trizma base)	1.8 g
Tricine	2.68 g
SDS	0.15 g

H_2O was added to a final volume of 150 ml.

2.12.2. Compositions of Tricine gels

(ml)	Separating gel*	Spacer gel	Stacking gel
Solution (A)		1.83	1.0
Solution (B)	3.0		
Solution (C)	3.0	3.0	3.1
H_2O		4.2	8.4
Urea	3.24 g		
APS (10%)	0.045	0.03	0.15
TEMED	0.0045	0.003	0.015

* Separating gel was made up to a final volume of 9 ml with H_2O.

2.12.3. Casting Tricine gels

Both the separating gel and the spacer gel were prepared simultaneously. The former was poured first and the latter was poured on top of the former. The two gels must be set together. The stacking gel was poured after both separating and spacer gel were set (Schagger *et al.*, 1987).

2.13. Isoelectric focusing (IEF)

2.13.1. Compositions of IEF rods

Urea	3.4 g
28.38% acrylamide/1.62% Bis	823 µl
10% Nonidet	1.237 ml
deionised water	1.1 ml
Ampholines pH 3.5 - 10	416 µl
TEMED	18.5 µl
10% APS	12.6 µl

2.13.2. Casting the IEF rod gels

The IEF glass rods were washed with chromic acid followed by rinsing with deionised water and then dried. Rods were sealed at one end with Parafilm. A mark was made with a maker pen at 10 cm from the sealed ends of the rods. Rods were placed in a clamp apparatus which held them in an upright position. The gel was poured into the rod from the unsealed end up to the mark. Urea (9M, 20 µl) was layered on top of the gel which was left for an hour to polymerise. The 9 M urea was removed and replaced with 20 µl of 2DMH (2DMH: 2% ampholines (pH 3.5 - 10), 300 mM NaCl, 1 mM EDTA, 2% Triton X-100, 5 mM ascorbic acid, 100 mM DTT in deionised water) + 9 M urea (60/40) and left for another hour. The parafilm seals were removed and replaced with dialysis membranes. During polymerisation, the cathode buffer (500 mM NaOH) and anode buffer (20 mM phosphoric acid) were made (Sinclair and Rickwood, 1982).

2.14. Electrophoresis conditions

2.14.1. Electrophoresis conditions for linear and gradient PAGE

Samples (40 µl) were either mixed with sample buffer (13.3 µl) described in section (2.8.1.1.1.) and boiled for 5 min in the case of SDS PAGE, or were mixed with loading buffer (30% glycerol and bromophenol blue) (10 µl) for native gels.
Electrophoresis was carried out using Bio-Rad power/pac 300 at constant current (5 mA for overnight or 45 mA for 3 hours). Electrophoresis was finished when the tracking dye reached the bottom of the gel.

2.14.2. Electrophoresis conditions for IEF

The IEF gels were prefocused at 200 V for 15 min followed by 300 V for 30 min and 400 V for 30 min. In the meantime, the samples were prepared and were mixed with 9 M urea and 2 DMH (30/30/40). After prefocusing, the cathode buffer was removed and replaced with prepared samples, overlaid with 2 DMH (10 µl). The electrophoresis was continued at 400 V for 12 hr followed by 1500 V for 1 hr.

2.14.3. Electrophoresis conditions for Tricine gel

Treatment of samples for Tricine gels was the same as that for SDS PAGE. The gels were run at 30 V constant voltage for 1 hour, followed by 50 to 100 mA constant current until tracking dye reached the bottom of the gel (Schagger *et al.*, 1987).

2.14.4. Molecular weight markers and pI markers

Electrophoresis calibration kits were supplied by Pharmacia (Uppsala, Sweden) or Sigma. Sigma low molecular weight calibration kit: albumin, bovine (66 kDa), albumin, egg (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).
Pharmacia high molecular weight calibration kit: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa), used for estimations of molecular weights of proteins resolved in native PAGE.
Pharmacia broad pI kit: amylglucosidase (3.50), methyl red dye (3.75), trypsin inhibitor (4.55), β-lactoglobulin A (5.20), carbonic anhydrase B, bovine (5.85), carbonic anhydrase B, human (6.55), myoglobin, acidic band (6.85) myoglobin, basic band (7.35), lentil lectin, acidic (8.15), lentil lectin, middle (8.45), lentil lectin, basic (8.65) and trypsinogen (9.30).

2.14.5. Visualisation of protein bands

Gels were stained in 45% methanol (v/v)/10% glacial acetic acid (v/v) containing Coomassie blue R-250 (2 g/l) for at least 10 min, followed by destaining in 20% methanol (v/v)/10% glacial acetic acid (v/v) in the case of slab gels (Hames and Rickwood, 1982). IEF rod gels were fixed in 10% TCA for 1 hr and 1 % TCA for another hour. After fixation, the rod gels were immersed in H_2O for a couple of hours, followed by staining and destaining procedures as described above.

2.14.6. Gel drying

Gels were mounted on two sheets of Whatman 3MM chromatography paper. The gels were then covered with a sheet of cling film which was trimmed to a size just larger than the gel. Gels were dried down for 40 min under vacuum at 80°C on a Bio-Rad dual temperature slab drier (Model 1125B).

2.15. Electrophoresis of nucleic acids —agarose gels

2.15.1. Solutions

(A) TBE (10X):

Tris base (Trizma base)	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml

H_2O was added to a final volume of 1000 ml.

(B) Ethidium bromide (10 mg/ml):

Ethidium bromide (1 g) was dissolved in H_2O (100 ml). The solution was stirred on a magnetic stirrer for several hours to ensure that the dye had dissolved. The container was wrapped in aluminium foil and stored at 4°C.

(C) Loading buffer:

Glycerol	3 ml
Bromophenol blue	0.25 g
Xylene cyanol	0.25 g

H_2O was added to a final volume of 10 ml, stirred, and kept at room temperature.

2.15.2. Preparation of agarose gel

The gel casting tray (Pharmacia, Uppsala, Sweden) was sealed with autoclave tape to make a mould. Agarose (Sigma, type II, 0.7 g) was dissolved in TBE (1X, 100 ml), heated and stirred until the agarose dissolved completely. The agarose was cooled down to about 50°C and ethidium bromide solution (0.025% (w/v)) was added to the agarose solution and stirred. The agarose solution was then poured into the mould. A suitable comb was inserted in the agarose which was left to gel (Maniatis *et al.,* 1982).

2.15.3. Electrophoresis conditions

Electrophoresis was performed using GNA-100 gel tank (Pharmacia, Uppsala, Sweden) at 80 mA. Electrophoresis was terminated when the first tracking dye neared the bottom.

2.15.4. Molecular weight markers

The following two sets of molecular markers were made using two different restriction enzymes. Bacteriophage DNA (phage λ, 10 μg), sterilised H_2O (90μl), and suitable buffer for the specific restriction enzyme (5 units) were very gently mixed together.

Restriction enzymes *HindIII* (5 units), and *PstI* (5 units) were individually added to phage λ DNA (10 μg) and incubated at 37°C for 2 hours to make two sets of molecular weight markers.

2.16. Transfer of proteins from PAGE to membrane

2.16.1. Blotting buffer

Blotting buffer was made up of 48 mM Tris, 39 mM glycine, 0.03% SDS and 10% methanol.

2.16.2. Transfer method

Blotting was carried out using a Bio-Rad blotting tank and power supply (Model 250/2.5). Two pieces of sponge and four sheets of 3 MM filter paper were immersed in the blotting buffer. A polyvinylidene difluoride (PVDF) membrane was cut out just bigger than the protein gel. The PVDF membrane was immersed in 100% methanol for 1 min and then immersed in the blotting buffer for 10 min. A piece of wet sponge was put on a blotting cassette. Two sheets of wet 3 MM filter paper were laid on the sponge. The PVDF membrane was laid on the filter paper. Protein gel was laid on the PVDF membrane. The other two sheets of wet filter paper were then laid on the gel. Another piece of sponge covered the filter paper. The blotting cassette was closed and inserted in the blotting tank in which the blotting buffer was poured. The membrane side faced the anode. Proteins were transferred onto the PVDF membrane from the gel by blotting at 250 mA for 2 hours (Juang, 1985 and Aebersold, 1989).

2.17. Transfer Nucleic acids from agarose gels to Nylon filter (Southern Blot)

2.17.1. Buffer

SSC (20X):

NaCl	175.3 g
Sodium citrate	88.2 g

Both solutes were dissolved in 800 ml of H_2O and the pH was adjusted to 7.0 using 10 N NaOH. The volume was adjusted to 1 l and the solution was sterilised by autoclaving.

2.17.2. Method

When electrophoresis was complete, the agarose gel was soaked in 0.25 M HCl for 30 min at room temperature to denature the DNA. The gel was then soaked in 0.5 M NaOH/1.5 M NaCl for 1 hour at room temperature with constant shaking. The gel was neutralised using 1 M Tris-HCl, pH 7.4/1.5 M NaCl for 1 hour at room temperature with constant shaking. An amount of SSC (20X) was poured into a baking dish. A glass plate was laid, spanning on the baking dish. An oblong shape piece of 3 MM paper was cut and laid on the glass plate. The filter paper must be long enough to touch the SSC (20X) buffer in the baking dish. Some SSC (20X) was poured on the filter paper to make it damp. The agarose gel was inverted so that its original underside was uppermost and laid on the damp filter paper. A high quality of nylon filter (Hybond-N) was trimmed as big as the gel and soaked in SSC (6X) for 2-3 min and then laid onto the gel. A wet piece of 3 MM paper just bigger than the gel was laid on the Hybond-N filter. A stack of paper towels (10 cm high) was placed on the 3MM paper. A glass plate was laid on the stack of

paper towels. A heavy weight was laid on the glass plate and left for overnight (Maniatis *et al.*, 1983).

2.18. DNA-DNA Hybridisation

2.18.1. Crosslinking

When the Southern blotting was finished, the Hybond-N membrane was soaked in SSC (6X) for 5 min at room temperature. Excess SSC (6X) was allowed to drain from the Hybond-N filter and it was dried on a sheet of 3MM paper at room temperature. The Hybond-N filter was removed to another sheet of 3MM paper (DNA side up). The crosslinking reaction was carried out using a crosslinker (UV Stratalinker type 2400, Stratagene, La Jolla, USA) for one cycle.

2.18.2. Hybridisation solutions

(A) Denhardt's solution (50X):

Ficoll	1 g
polyvinylpyrrolidone	1 g
BSA (fraction V)	1 g

The solutes were dissolved in H_2O to make final volume of 100 ml.

(B) OLB buffer:

solution (a) Tris-HCl buffer (1.25 M., pH 8.0, 0.125 M $MgCl_2$) (1 ml) containing 2-mercaptoethanol (18µl) and dXTP (5 µl each of 0.1 M dATP, 0.1 M dTTP and 0.1 M dGTP in TE buffer).

solution (b) 2M HEPES, pH 4.0.

solution (c) Hexadeoxynucleotides (P.L. #2166) (50 OD units was suspended in 550 µl TE buffer to give concentration of 90 OD/ml).

Solution (a), (b) and (c) (10:25:15) (50 µl) was mixed thoroughly.

(C) SSPE (20X)

NaCl	174 g
$NaH_2PO_4.H_2O$	27.6 g
EDTA	7.4 g

The solutes were dissolved in 800 ml of H_2O and the pH was adjusted to 7.4 using 10 N NaOH. The volume was adjusted to 1 litre and the solution was sterilised by autoclaving.

2.18.3. Hybridisation

The crosslinked Hybond-N filter was removed to a heat-sealable plastic bag. Prehybridisation solution consisting of SSPE (6X), 0.5 % SDS and Denhardt's solution (5X) was pre-warmed at 55°C and poured into the plastic bag which contained the filter. The air was squeezed from the plastic bag and the open end was sealed, followed by incubation at 55°C for 3 hr. The probe (β-phycocyanin gene from *Synechococcus* sp. PCC 7002) was labelled using random priming or nick translation by following the manufacturers' instructions. Herring sperm DNA (10 mg/ml, 100µl) and TE buffer (80µl) were added to the probe, which was heated at 100°C for 10 min and then kept on ice for several minutes. After prehybridisation a corner of the plastic bag was cut off to make a small hole. The labelled probe was added into the plastic bag through the small hole, and the plastic bag was resealed, followed by hybridisation at 55°C overnight. The filter was washed twice with SSC (5X) containing 0.1 % SDS at 55°C, 10 min. The hybridisation

solution was kept at -20°C. The filter was removed to another plastic bag, sealed and subjected to autoradiography.

2.19. N-terminal amino acid sequence analysis

2.19.1. Protease digestion

N-terminal amino acid sequence analyses were conducted at the Protein Sequencing Facility at Leicester University. Protein bands of interests were cut out of a preparative SDS gel, followed by electroelution (Legendre and Matsudaira, 1989). The electroeluted protein was concentrated under a stream of nitrogen. The concentrated protein was digested using a suitable protease or more than one protease. The protease digest fragments were separated by Tricine gel electrophoresis (section 2.8.1.4.). The Tricine gel was blotted onto a PVDF membrane (section 2.9.1.2.), stained and subjected to autoradiography in the case of ^{32}P labelled protein (Legendre and Matsudaira, 1989). The PVDF membrane was air dried and protein bands of interest were marked with a pencil, prior to being sent to Leicester University to be N-terminally sequenced.

2.19.2. Cyanogen bromide (CNBr) cleavage

Protein bands of interest were cut out of a preparative gel, followed by electroelution. The eluted protein was concentrated (final volume 100 µl). Formic acid (70%, 200 µl) was added in the protein solution, followed by addition of solid CNBr (3 mg) and mixed thoroughly. The protein solution was kept at 0-4°C for overnight. The protein sample was dried down using vacuum centrifugation to get rid of the CNBr and formic acid. Water (100 µl) was added to the protein sample and vacuum centrifuged again to remove the CNBr remaining in the protein sample.

2.20. Localisation of phosphorylation site

2.20.1. Hydrolysis of protein

A protein gel was blotted onto a PVDF membrane, followed by staining and/or autoradiography. The bands of interest were cut out and removed to an Eppendorf tube to which 300 µl 6 M HCl was added and hydrolysed at 110-120°C in an autoclave for 2 hr. The PVDF membranes were removed and the sample solution was dried down under a stream of nitrogen (Mann *et al.*, 1991). The dried amino acids were dissolved in 10 µl of H_2O.

2.20.2. Thin layer chromatography (TLC)

A dot 1 cm from the right edge and the bottom edge of a cellulose pre-coated HPTLC plate (10 x 10 cm; Merck, Germany) was made with a pin. Amino acids (1 mg/ml) derived from protein molecules were pipetted in small aliquots (0.2 µl) onto the dot and dried using a hair dryer. The solvent for separation of different phosphoamino acids consisted of iso-butanol/concentrated HCl/H_2O (75/15/15). The TLC plate was developed overnight and was then taken out from the tank and removed to fumehood and dried. The plate was developed with ninhydrin (ninhydrin/acetone 0.5% (w/v)) and heated (60°C) until the amino acids were visible.

2.20.3. Localisation of phosphorylation site of phosphoproteins

The phycobiliprotein-containing fractions eluted from an FPLC Mono Q column were collected (section 2.11.1.), labelled with [γ-^{32}P] ATP and digested with a suitable protease, followed by separation using an HPLC reverse phase C8 column (100 x 4.6 mm, pore size 90 Å, Bio-Rad Lab., CA, USA) (Stone *et al.*, 1989).

2.21. Protein purification

2.21.1. Anion exchange chromatography

Anion exchange chromatography was performed using an FPLC Mono Q 5/5 column (Pharmacia, Uppsala, Sweden). The Mono Q column was equilibrated with approximately 10 column volumes of 20 mM Tris.HCl buffer, pH 7.5, until the base line was stable. Proteins were applied to the column and eluted using a linear 0-1 M KCl gradient. Fractions (1 ml) were collected, and subsequently assayed for kinase activity using the *in vitro* assay described in section (2.3.5.).

2.21.2. Concentrating protein

Protein concentration was performed using either an ultrafiltration cell (Amicon, Danvers, USA). The protein solution was poured into an Amicon ultrafiltration cell on a magnetic stirrer. The protein solution was concentrated under a stream of nitrogen to reduce the amount of the TES buffer in the protein solution. Alternatively, protein concentration was also performed by precipitation of proteins with 90% saturated ammonium sulphate. Aliquots of the protein solution were mixed with 100% saturated of ammonium sulphate solution to make 90% saturated solution. The precipitated proteins

were collected by centrifugation at 22,000 x g (Beckman JA17 rotor), 4°C for 20 min. The protein pellets were dissolved in small amounts of TES buffer.

2.21.3. Gel filtration chromatography

Gel filtration chromatography was performed using an FPLC Superose 12 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 20 mM Tris.HCl buffer containing 0.1 M KCl, pH 7.5. Protein solutions were applied to the Superose column. Fractions (1 ml) were collected, and subsequently assayed for kinase activity using the *in vitro* assay described in section (2.3.5.).

2.21.4. Desalting protein solution

The desalting of protein solutions was performed using a PD-10 Sephadex G-25M column (Pharmacia, Uppsala, Sweden). The PD-10 column was equilibrated with approximately 5 column volumes of buffer. Aliquots (2.5 ml) of protein solution were applied to the column. Protein solution was collected (3.5 ml) followed by assay for kinase activity (section 2.3.5.).

2.22. Spectroscopy

2.22.1. Absorption spectra

Aliquots (36µl) of β-phycocyanin complex were added to TES buffer (1 ml), mixed and then removed to cuvettes (1 ml). The absorption spectra were read at between 550 and 650 nm. The absorption was carried out using a scanning spectrophotometer (type PU 8720 UV/VIS, Philips).

2.22.2. Fluorescence emission spectra

Aliquots (36 µl eqivalent to 50 µg of proteins) of β-phycocyanin complex were added to TES buffer (3 ml), mixed and then removed to cuvettes (3 ml). The fluorescence spectra were recorded using a Perkin-Elmer LS-5 fluorescence spectrometer. The excitation wavelength was set at 600 nm, and the fluorescence emission spectra were read between 620 and 720 nm.

2.23. Phosphorylation and dephosphorylation of proteins under illumination of different qualities of light

2.23.1. Protein phosphorylation and dephosphorylation in the orange light or in the blue light

Aliquots (36 µl eqivalent to 50 µg of protein) of the phycobiliprotein-containing fractions were added into an Eppendorf tube, which was wrapped in an orange or a blue plastic (Lee Filters, England) and incubated at 20°C for 40 min. The blue filter transmits light greater than 700 nm which is predominantly absorbed by PS I. The orange filter transmits light with wavelength between 550 nm to 620 nm, which is predominantly absorbed by PS II. (N. J. Silman, personal communication). Proteins were then subjected to *in vitro* phosphorylation in the blue light or orange light.

2.23.2. Light-dark shifts of protein phosphorylation

Aliquots (36μl eqivalent to 50 μg of proteins) of the phycobiliprotein-containing fractions of the sucrose gradient were added into a cuvette and illuminated with light with wavelength of 580 nm (PS II light) or 700 nm (PS I light) for 40 min. The illuminated phycobiliprotein-containing fractions of the sucrose gradient were then removed to an Eppendorf tube. Proteins were then subjected to *in vitro* phosphorylation in white light or in the dark for 10 min.

2.24. Gene cloning

2.24.1. Cosmid library

Both *Synechocystis* sp. PCC 6803 chromosomal DNA and the cosmid library (a kind gift of Prof. S. Shestakov of Moscow State University) were probed with the β-phycocyanin gene from *Synechococcus* sp. PCC 7002. Positive cosmid clones were grown in LB medium containing kanamycin (25 μg) (Km 25). Extraction of the cosmid DNA was carried out by the miniprep method. The β-phycocyanin gene in the cosmid clones was amplified using the polymerase chain reaction (PCR).

2.24.2. Miniprep of DNA

Cells were harvested by centrifuged at 10,000 x g for 5 min and resuspended in 100 μl of ice-cold digestion solution containing lysozyme (5 mg/ml), 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0. Cells were transferred to an Eppendorf tube and stored on ice for 5 min. Freshly prepared lysis mixture (0.2 M NaOH, 1% SDS (w/v), 200 μl) was added to the cells. The Eppendorf tube was inverted for 2-3 times, stored on ice for further 5 min. 150 μl of ice-cold potassium acetate pH 4.8 solution (60 ml, 5 M

potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O) were added to the cells. After vigorous vortexing, the Eppendorf tube was stored on ice for further 5 min and centrifuged for 5 min in an Eppendorf centrifuge. The supernatant was transferred to a fresh Eppendorf tube, an equal volume of phenol/chloroform added, mixed by vortex and centrifuged in an Eppendorf centrifuge for 4 min. The supernatant was transferred to a fresh tube and 2 volumes of ethanol was added to precipitate the DNA.

2.24.3. Polymerase Chain Reaction (PCR)

The PCR was performed using a DNA Thermal Cycler (Perkin-Elmer, Norwalk, USA)
Composition of the PCR reaction:

Buffer for *Taq* polymerase (10X)	5 µl
5 mM dNTP	2 µl
50 mM $MgCl_2$	1.5 µl
primer I (forward primer)	5 µl
primer II (reverse primer)	5 µl
template	1 µl
Taq polymerase *	0.25 µl
sterilised H_2O	30.25 µl

**Taq* polymerase was not added in the mixture until required.

All the reagents were added in sterilised Eppendorf tubes (0.5 ml) and mixed together (except *Taq* polymerase), overlayered with liquid paraffin (30 µl) and kept in 92°C for 3 min. The temperature was lowered down to 80°C and *Taq* polymerase was added. A cycle of PCR consisted of 92°C for 60 sec, 40°C for 60 sec and 72°C for 60 sec. PCR

was repeated for 30 cycles, followed by 72°C for 10 min. When the PCR was finished, the Eppendorf tubes were stored at 4°C for future use.

Chapter 3

Protein Phosphorylation in *Synechocystis* sp. PCC 6803

3.1. Introduction

That thylakoid membranes possess protein kinase activities was first demonstrated by illumination of pea chloroplasts supplied with [^{32}P]-orthophosphate (Bennett, 1977). Light-dependent kinase activity was also demonstrated *in vitro*, where reducing agents were capable of activating a protein kinase in the dark (Bennett, 1979a; for review see Bennett, 1991). Protein kinase activities have also been demonstrated in higher plant chloroplasts, and their role in state-transitions has been widely accepted (for review see Bennett, 1991; Allen, 1992).

Cyanobacteria have also been shown to exhibit state-transitions with the concomitant phosphorylation of a number of thylakoid proteins (Sanders and Allen, 1987, and 1988; Harrison *et al.*, 1991; Race and Gounaris, 1993). Besides, protein phosphorylation has also shown to be involved in the adaptation to environmental stimuli in cases such as nitrogen depletion, bicarbonate concentration and salt shock in cyanobacteria (Harrison *et al.*, 1990; Tsinoremas *et al.*, 1991; Forchhammer and Tandeau de Marsac, 1994; Bloye *et al.*, 1992; Hagemann *et al.*, 1993).

Consequently, the overall aim of this project was to establish some aspects of the physiological roles of protein phosphorylation in cyanobacteria, especially focusing on the relationship between protein phosphorylation and photosynthesis. The first part of the project involved studies of protein phosphorylation *in vitro* and identification of a phosphorylated protein(s). The strategy was to establish conditions *in vitro* where we could get significant phosphorylation of the proteins associated with the photosynthetic apparatus.

3.2. *In vitro* protein phosphorylation

It has been demonstrated that the thylakoid membranes possess protein kinase activities (Bennett, 1977) and some proteins associated with the thylakoid membranes have been found to be phosphorylated both *in vivo* and *in vitro* (Bennett, 1977; Bennett, 1979a; Sanders and Allen, 1987, and 1988; Harrison *et al.*, 1991; Race and Gounaris, 1993) Therefore, the cyanobacterial thylakoid membranes were used for research on the phosphorylation of proteins. Several kind of buffer systems have been used for experiments on protein phosphorylation, such as HEPES (Harrison *et al.*, 1991) and TES (Murata and Omata, 1988). TES buffer has been used for the preparation of thylakoid membranes from *Synechocystis* sp. PCC 6803 (Murata and Omata, 1988) and hence TES buffer was used throughout this project unless otherwise stated. The thylakoid membranes represent most of the total membrane (Allen, 1968); therefore, total membranes were prepared by ultracentrifuging cell-free extracts of *Synechocystis* sp. PCC 6803. After ultracentrifugation, the pellet of total membranes would be mainly composed of the thylakoid membranes which were pelleted at 15,000 x g, and cytoplasmic membranes and cell walls (Allen, 1968; Murata and Omata, 1988). Because the densities of the thylakoid membranes, cytoplasmic membranes and cell walls are different, a density gradient centrifugation can be used to separate them. Three commonly used nonionic media are Percoll, Ficoll and sucrose; the viscosity of Percoll and Ficoll are too high to make a gradient suitable for separating the thylakoid membranes. By contrast, the viscosity of sucrose is lower and the density of the same percentage of sucrose solution (w/v) falls between that of Percoll and that of Ficoll and is therefore suitable for the separation of the thylakoid membranes (Rickwood, 1984). Hence, in order to separate the thylakoid membranes, the total membranes were subjected to sucrose density gradient centrifugation. After ultracentrifugation, the orange-coloured, undigested cell walls were located in the 80% sucrose band (Fig. 3.1) (Murata *et al.*,

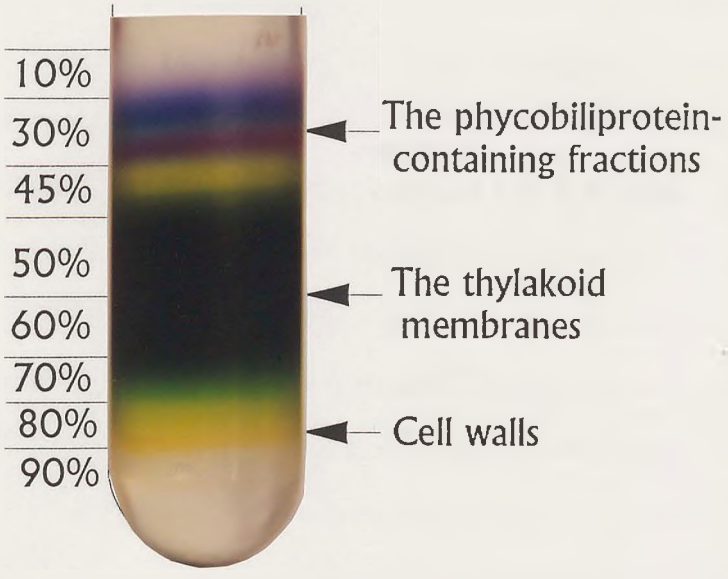

Fig. 3.1. The sucrose density gradient of *Synechocystis* sp. PCC 6803 total membranes.

Synechocystis sp. PCC 6803 cells were broken by passage through a French pressure cell. The cell-free extracts subjected to ultracentrifugation. The pellet consisting of total membranes, was subjected to sucrose density gradient centrifugation at 4°C for 18 hr. The thylakoid membranes, the phycobiliprotein containing fractions and the cell walls are indicated.

1981; Murata and Omata, 1988; Hoiczyk and Baumeister, 1995). The thylakoid membranes, because the density of the thylakoid membranes is 1.17-1.18 (g/ml), were found in the 60% to 50% of the sucrose region and could be identified by their green colour (Fig. 3.1) (Murata and Omata, 1988). Two blue bands located in the 30% sucrose fractions contained phycobiliproteins (Fig. 3.1) because proteins in these fractions showed a broad absorption spectra with a maximum at 620 nm, corresponding to the phycocyanobilin, and SDS-PAGE analysis together with UV light illumination showed proteins in these phycobiliprotein-containing fractions to emit strong fluorescence. In order to investigate protein phosphorylation *in vitro* and since proteins kinase activities have been demonstrated in the cell-free extracts of several cyanobacterial strains (Harrison *et al.*, 1991; Mann *et al.*, 1992; Silman *et al*, unpublished data), proteins in the soluble fractions (the supernatants of ultracentrifugation for pelleting the total membranes) and those in different fractions of the sucrose gradient were subjected to *in vitro* phosphorylation by addition of [γ-^{32}P]ATP (5 μCi/μmole) in the presence of $MgCl_2$/ATP (final concentration: 5 μM $MgCl_2$ and 0.5 μM ATP) and incubation at 25°C for 60 min. The proteins were then separated by SDS-PAGE and subjected to autoradiography which revealed that some proteins occurring in the soluble fraction, and the thylakoid membrane fractions (60-50% sucrose region) and the phycobiliprotein-containing fractions (30% sucrose band) of the sucrose density gradient were [^{32}P]-labelled (Fig. 3.2). Treatment of the SDS-PAGE gel with hot TCA did not cause loss of radiolabelling, suggesting that the proteins were likely to be phosphorylated and the phosphorylated proteins contained phosphate monoester linkages rather than phosphoramidates or acylphosphates as these are both labile under acid conditions (data not shown) (Manai and Cozzone, 1982). Treatment with protease K destroyed all the radiolabelled bands, indicating that they were all proteins rather than polyphosphate or nucleic acids (data not shown) (Yang *et al.*, 1992). Therefore, all the results confirmed

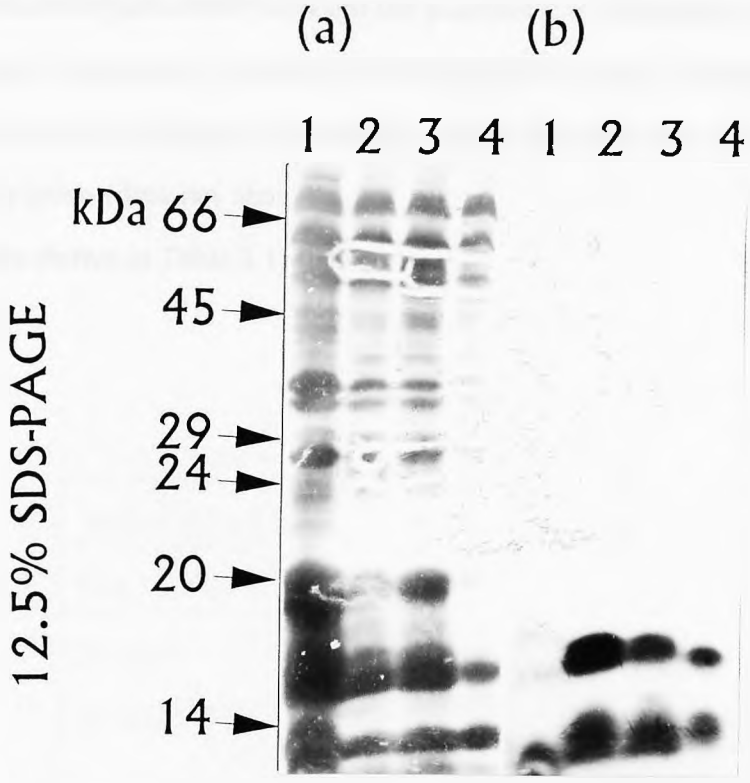

Fig. 3.2. Protein phosphorylation in different fractions of cell-free extracts of *Synechocystis* sp. PCC 6803.

Proteins in each fraction of the sucrose gradient were subjected to *in vitro* phosphorylation with addition of [γ-32P]ATP (5 μ*Ci*) and incubated at 30°C for 60 min, followed by SDS-PAGE analysis and autoradiography. (a) A Coomassie blue stained 12.5% SDS-PAGE gel; (b) Autoradiograph. Each track is as follows. Track 1, soluble fraction; track 2, upper part of the phycobiliprotein-containing fraction (30% sucrose band); track 3, lower part of the phycobiliprotein-containing fraction (30% sucrose band); track 4, the thylakoid membrane fraction.

proteins as being phosphorylated and revealed the existence of protein kinase activities in the soluble fractions, the thylakoid membranes and the phycobiliprotein-containing fractions of the sucrose gradient. Kinase-free phosphorylation has been reported (Vener, 1989); however, our experiments ruled out the possibility of kinase-free phosphorylation, since no protein was phosphorylated when the phycobiliprotein-containing fractions of the sucrose gradient were heated and proteins in these fractions were then subjected to *in vitro* phosphorylation (data not shown). The most prominent phosphorylated proteins in each fraction are shown in Table 3.1.

Table 3.1

Molecular masses of phosphoproteins in different fractions of cell-free extracts of *Synechocystis* sp. PCC 6803. The cell-free extracts were subjected to centrifugation to removed the unbroken cells and cell debris. The supernatant were collected and subjected to ultracentrifugation at 4°C for 1 hr. The soluble fractions (supernatant) were frozen at -20°C for future use. The total membranes (pellets) were layered onto a sucrose density gradient and ultracentrifuged at 4°C for 18 hr. After ultracentrifugation, two phycobiliprotein-containing fractions (30% sucrose bands) and the thylakoid membranes were separated.

Soluble fractions	Total membranes (then subjected to a sucrose gradient)		
	phycobiliprotein-containing fractions		the thylakoid membranes
	10% sucrose band	30% sucrose band	
14 kDa*	14 kDa*	14 kDa*	14 kDa*
18 kDa	18 kDa	18 kDa	18 kDa
19 kDa			
	20 kDa*	20 kDa*	20 kDa*
	56 kDa*		

* Phosphorylation of the 14 kDa, the 20 kDa and the 56 kDa proteins occurred inconsistently from experiment to experiment.

Besides the strongly phosphorylated proteins shown in Table 3.1, several other phosphoproteins could also be found in the phycobiliprotein-containing fractions (30% sucrose band) of the sucrose gradient. It was noticeable that two phosphoproteins with molecular masses of 14 kDa and 18 kDa were located in both the thylakoid and phycobiliprotein-containing fractions; however, phosphorylation of this 14 kDa protein did not always occur. In addition, 20 kDa and 56 kDa proteins were also phosphorylated and the phosphorylation event, like that of the 14 kDa protein, was inconsistent from experiment to experiment. However, it is still unknown why these proteins could not be consistently phosphorylated in every experiment; even when the same experiments with the same samples were repeated. By contrast, phosphorylation of the 18 kDa was a very consistent phosphorylation reaction. Under UV light illumination, an 18 kDa protein showed strong fluorescence (Fig. 3.3). Therefore, this 18 kDa protein was thought to be one of the components of the phycobilisomes because phycobiliproteins absorb UV light and strongly fluoresce (Glazer, 1989). In the thylakoid membranes of *Synechococcus* sp. PCC 6301 an 18.5 kDa protein was found to be phosphorylated and was one of the components of phycobilisomes (Sanders and Allen, 1987), and this protein was later on tentatively identified as β-phycocyanin (Harrison, 1990). In our experiments, the 18 kDa protein, a component of the phycobilisomes, was consistently and strongly phosphorylated; therefore, the phosphorylated 18 kDa protein was chosen as the target of future research. The 18 kDa protein was abundant in the phycobiliprotein-containing fractions of the sucrose gradient, which would also facilitate future experiments.

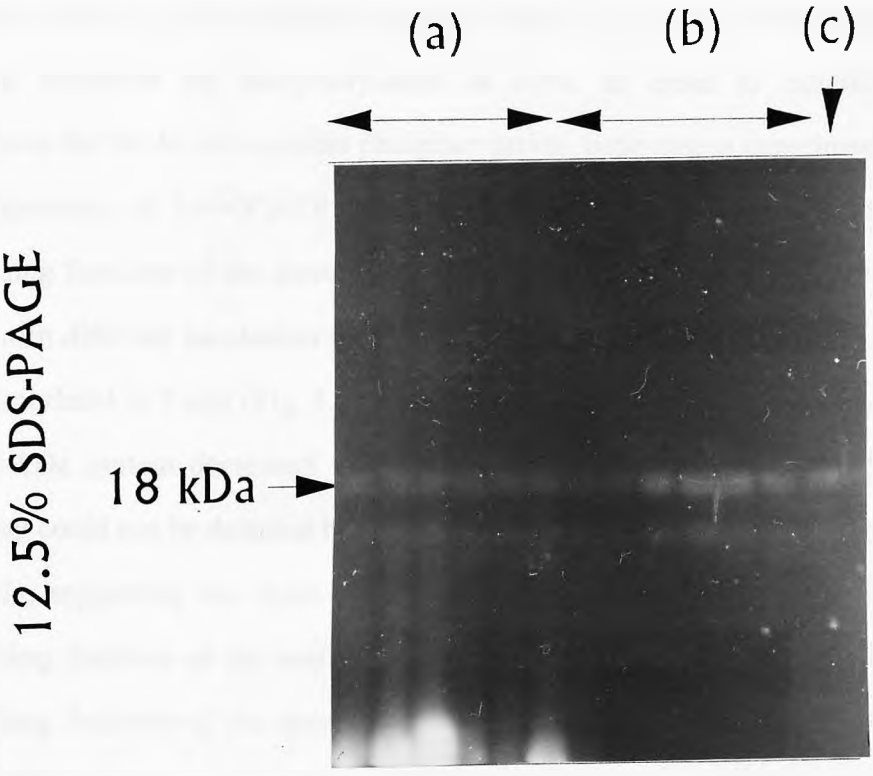

Fig. 3.3. Fluorescence of phycobiliproteins.

Proteins in each fraction (1 ml) of the sucrose gradient were subjected to *in vitro* phosphorylation and separated by a 12.5% SDS gel. The SDS-PAGE gel was then placed on a transilluminator and illuminated with UV light. The phycobiliproteins, due to the chromophores, the bilin groups, were visualised by UV light illumination. (a) the thylakoid membrane fractions and (b) the phycobiliprotein-containing fractions of a sucrose gradient. (c) the soluble fractions, the supernatant of ultracentrifugation for pelleting the total membranes.

3.3. Optimal conditions for *in vitro* phosphorylation

Phosphorylation of proteins *in vitro* had been demonstrated in the previous section; however, the conditions for the phosphorylation of proteins might not be the optimal condition for phosphorylation *in vitro*. In order to establish the optimal conditions for the *in vitro* protein phosphorylation, time course experiments and different concentrations of [γ-^{32}P]ATP were investigated. Proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation at 25°C with different incubation times. These assays showed that the 18 kDa protein was phosphorylated in 5 min (Fig. 3.4). It was also noticeable that the intensity of labelling of the 18 kDa protein decreased when the incubation time increased to 30 min and the labelling could not be detected by autoradiography when the incubation time increased to 960 min, suggesting that there might be phosphatase activities in the phycobiliprotein-containing fractions of the sucrose gradient. When the proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation with different concentrations of [γ-^{32}P]ATP (5-25 $\mu Ci/\mu$mole) (final concentration) at 25 °C for 30 min, no significant difference in the labelling of the 18 kDa protein were detected (Fig. 3.5). $MgCl_2$ was used in the assay of *in vitro* phosphorylation because protein kinases require Mg^{2+} which forms a complex with ATP. It has been reported that concentration of Mg^{2+} also affected phosphorylation reaction because the stability constant (the association constant of the metal-ligand complex) suitable for one kinase might not be suitable for another (Dixon *et al.*, 1979). However, our experiments showed that 5 μM $MgCl_2$ (final concentration) was suitable for the *in vitro* phosphorylation. The use of the unlabelled ATP (0.5 μM) was to prevent the non-specific labelling because without the addition of the unlabelled ATP in the *in vitro* phosphorylation assay, non-specific labelling was normally a problem. However, why the addition of the unlabelled ATP could reduce the non-specific labelling is not well understood. According to the

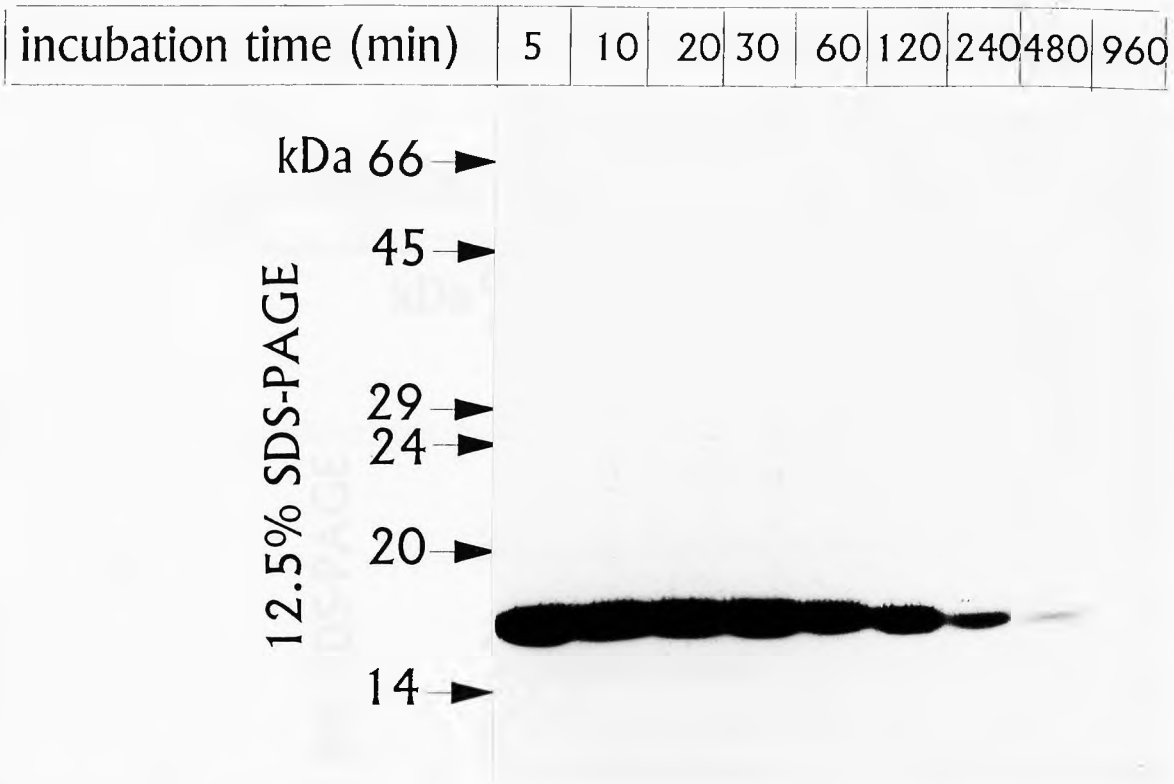

Fig. 3.4. Time course of phosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose gradient.

Proteins in the phycobiliprotein-containing fractions of a sucrose gradient were subjected to *in vitro* phosphorylation. Incubation times of the *in vitro* phosphorylation reaction are as indicated. Intensity of the radioactivity was detected by autoradiography.

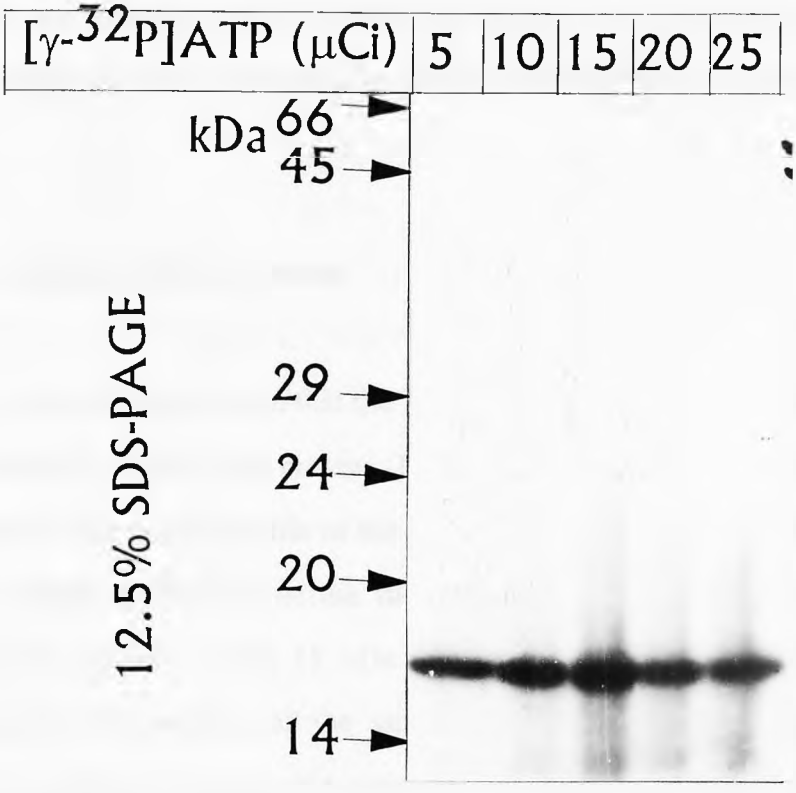

Fig. 3.5. Protein phosphorylation with different concentrations of [γ-^{32}P]ATP.

The phycobiliprotein-containing fractions of the sucrose gradient were pooled together and proteins were subjected to *in vitro* phosphorylation with addition of different concentrations of [γ-^{32}P]ATP which are as indicated and incubated at 30°C for 60 min. The radioactivity was detected by autoradiography.

results presented in these experiments, conditions for *in vitro* phosphorylation were therefore established at incubation of the phycobiliprotein-containing fractions of the sucrose gradient by the addition of [γ-^{32}P]ATP (5 μCi/μmole, final concentration) in the presence of 5 μM $MgCl_2$ (final concentration) and 0.5 μM ATP (final concentration) at 25°C for 5 min.

3.4. Identifying a phosphorylated protein

Previous experiment had shown that the 18 kDa was probably a phycobiliprotein; therefore, we wished to identify this protein. There are two stages to reach this goal. Firstly, comparison of the protein profile of the isolated phycobilisomes with that of the phycobiliprotein-containing fractions of the sucrose gradient, and comparison the N-terminal amino acid sequence of the 18 kDa protein with those of known proteins. Secondly, cloning and sequencing of the gene encoding the 18 kDa protein and comparison of the predicted amino acid sequence with the N-terminal amino acid sequence of the tentatively identified 18 kDa protein to see whether or not the amino acid sequences are identical. The second stage will be included in Chapter 5.

3.4.1. Comparison of protein profile of the phycobiliprotein-containing fractions of the sucrose gradient with that of the isolated phycobilisomes

In order to compare the protein profile of the phycobilisomes with that of the phycobiliprotein-containing fractions of the sucrose gradient, isolation of the intact phycobilisomes was performed. High concentrations of phosphate buffer (0.75 M NaK_2PO_4, pH 7.5), appropriate temperature control (20-22°C) and avoidance of light are essential conditions for isolation of intact phycobilisomes (Glazer, 1988a). Thylakoid membranes associated with intact phycobilisomes were obtained by breaking the cells,

followed by ultracentrifugation (Elmorjani *et al.*, 1986; Glazer, 1988a). After being released from the thylakoid membranes by 1% Triton X-100 (final concentration), the intact phycobilisomes were subjected to a sucrose density gradient centrifugation to separate the phycobilisomes from the soluble proteases (Elmorjani *et al.*, 1986). After centrifugation, the intact phycobilisomes are located in the lower part of the 0.75 M sucrose band (Elmorjani *et al.*, 1986) (Fig. 3.6). The isolated intact phycobilisomes were subjected to SDS-PAGE analysis to compare the protein profile of phycobilisomes and that of the phycobiliprotein-containing fractions of the sucrose gradient. Besides, C-phycocyanin from red alga *Porphyra tenera*, which is commercially available and that from *Synechococcus* sp. PCC 7942 (a kind gift of Dr. D. Clarke) were also obtained for comparison of these protein profiles. An autoradiograph of the [^{32}P]-labelled 18 kDa protein together with the comparison of the protein profiles showed that the phosphorylated 18 kDa protein migrated with the same size as β-phycocyanin (Fig. 3.7), suggesting that the 18 kDa protein could well be β-phycocyanin.

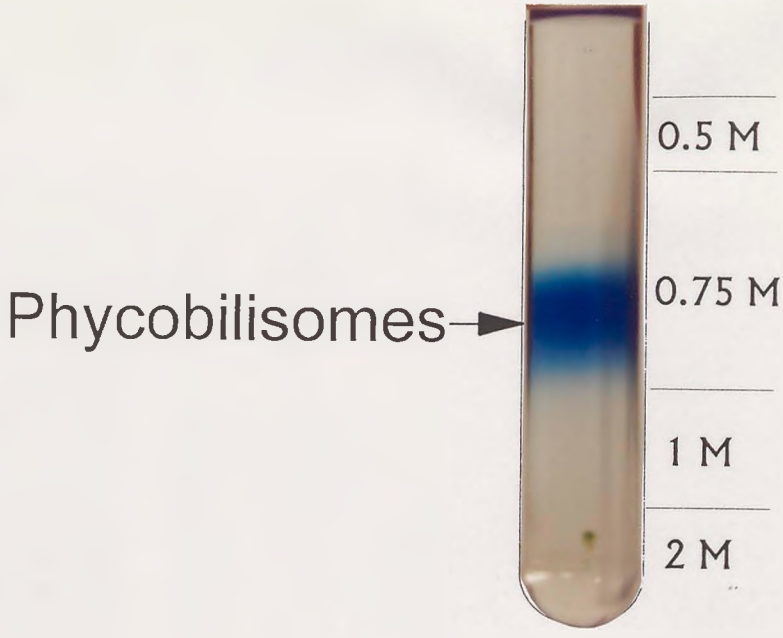

Fig. 3.6. Isolation of phycobilisomes.

Synechocystis sp. PCC 6803 cells were washed with 0.75 M NaK_2PO_4 buffer, pH7.5 and then broken by passage through a French pressure cell. The cell-free extracts were subjected to ultracentrifugation to pellet the total membranes (mainly thylakoid membranes). The thylakoid membranes carrying intact phycobilisomes were incubated with Triton X-100 (1%) at 20°C for 30 min and then subjected to ultracentrifugation. The supernatants contained intact phycobilisomes. The supernatants were subjected to a sucrose density gradient centrifugation. The intact phycobilisomes, are located in the lower part of 0.75 M sucrose band (Elmorjani *et al.*, 1986).

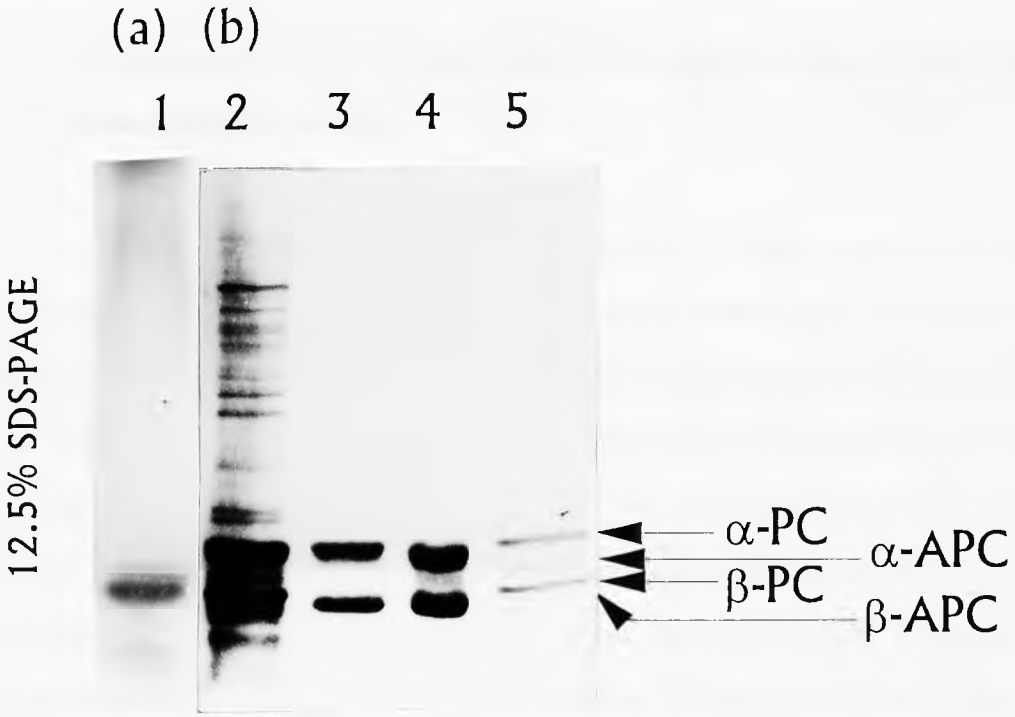

Fig. 3.7. Protein profile of the phycobiliprotein-containing fractions and that of the isolated phycobilisomes

Proteins in the phycobiliprotein-containing fractions of a sucrose gradient were subjected to *in vitro* phosphorylation. Proteins in the phycobiliprotein-containing fractions of the sucrose gradient and the isolated phycobilisomes were analysed by a 12.5% SDS PAGE gel. (a) autograph. (b) Coomassie blue stained gel. Track 1 is autoradiograph of track 2, protein profile of the phycobiliprotein-containing fractions of the sucrose density gradient. Track 3, C-phycocyanin, consisting of both α- and β-phycocyanin from *Porphyra tenera* (purchased from Sigma Co.) and track 4, C-phycocyanin from *Synechococcus* sp. PCC 7942; track 5, isolated phycobilisomes of *Synechocystis* sp. PCC 6803.

Abbreviations: α-PC, α-phycocyanin; α-APC, α-allophycocyanin; β-PC, β-phycocyanin; β-APC, β-allophycocyanin.

3.4.2. Comparison of the N-terminal amino acid sequence of the 18 kDa with those of known proteins

Previous experiments had shown that the 18 kDa protein was probably a phycobiliprotein, possibly β-phycocyanin. In order to confirm this, comparison of the N-terminal amino acid sequence of the 18 kDa protein against proteins in a protein database was performed as follows. Proteins in the phycobiliprotein-containing fractions of a sucrose density gradient were subjected to *in vitro* phosphorylation, followed by SDS-PAGE analysis. The phosphorylated 18 kDa protein band was cut out of the gel and electroeluted, and the eluted 18 kDa protein was then digested with *Staphylococcal* V8 protease (hereafter referred to as V8 protease). The V8 protease digestion fragments were separated by a Tricine gel, blotted onto a PVDF membrane and subjected to autoradiography. Autoradiography showed that a 9 kDa protein band of the V8 digestion fragment was found to be [^{32}P]-radiolabelled and the PVDF membrane was sent to Leicester University for N-terminal amino acid sequencing. The N-terminal amino acid sequence of this 9 kDa fragment was VFDVFTRVVSQADA and showed 85.7% similarity to β-phycocyanin from *Synechococcus* sp. PCC 7002 and several cyanobacterial strains (Fig. 3.8). Since the 9 kDa protein was the V8 protease digestion of the 18 kDa protein band cut out of the SDS-PAGE gel, and there was only one N-terminal amino acid sequence of this 9 kDa fragment, this 9 kDa protein should be rather pure. Even though more than one protein having the same N-terminal amino acid sequences might occurred in the same system, the probability was very low, especially when protein band was cut out of an SDS-PAGE gel, followed by protease digestion of this protein band. Therefore, the 18 kDa protein was tentatively identified as β-phycocyanin. This sequence corresponds to residues 1 to 14 of known β-phycocyanin and suggested that the phosphorylation site was located in the N-terminal 9 kDa fragment of the 18 kDa protein.

```
          1                                                    50
6701pcb   .MYDAFTRVV SQRDARGEFL SSAQIDALSK LVSDSNKRID TVNRITGNAS
7120pcb   MTLDVFTKVV SQADSRGEFL SNEQLDALAN VVKEGNKRLD VVNRITSNAS
 dc2pcb   .MFDAFTKVV AQADARGQFI SASEIDALAA MVSDSNKRLD AVNRISSNAS
7002pcb   .MFDIFTRVV SQADARGEFI SSDKLEALKK VVAEGTKRSD AVSRMTNNAS
9 kDa pep .VFDVFTRVV SQADA

          51                                                   100
6701pcb   AIVTNAARSL FAEQPQLIAP GGNAYTSRRM AACLRDMEII LRYVTYAIFA
7120pcb   AIVTNAARAL FEEQPQLIAP GGNAYTNRRM AACLRDMEII LRYVTYAILA
 dc2pcb   TIVASAARQL FAQQPSLIAP GGNAYTSRRM AACLRDMEII LRYVTYASFA
7002pcb   SIVTNAARQL FADQPQLIAP GGNAYTNRRM AACLRDMEII LRYVTYATFT

          101                                                  150
6701pcb   GDASVLEDRC LNGLRETYLA LGTPGSSVAV GVQKMKDEAL AIANDTNGIT
7120pcb   GDASVLDDRC LNGLRETYQA LGTPGSSVAV GVQKMKDAAV GIANDPNGIT
 dc2pcb   GDASVLEDRC LNGLRETYLA LGTPGASVAA GVNLMKESAL AIVNDRAGIS
7002pcb   GDASVLNDRC LNGLRETYVA LGVPGASVAA GVRAMGKAAV AIVMDPAGVT

          151                   174
6701pcb   LGDCSALMAE VATYFDRAAA AVA*
7120pcb   KGDCSQLISE VACYFDRAAA VG*.
 dc2pcb   AGDCASLSSE IGTYFDRAAA AVA*
7002pcb   SGDCSSLQQE IELYFETAAK AVE*
```

Fig. 3.8. The amino acid sequences of β-phycocyanin from different cyanobacterial strains (Adapted from a protein database).

Abbreviations: 6701, *Synechocystis* sp. PCC 6701; 7120, *Synechococcus* sp. 7120; dc2, *Synechococcus* sp. WH 7803; 7002, *Synechococcus* sp. 7002; pcb, β-phycocyanin; 9 kDa pep, the N-terminal amino acid sequences of the 9 kDa fragment of the V8 protease digested 18 kDa protein.

3.5. Localisation of phosphorylation site in β-phycocyanin

Since the 18 kDa protein was tentatively identified as β-phycocyanin, we also wished to know which amino acid residue(s) was phosphorylated. As long as the phosphorylation site is known, site-directed mutation(s) leading to strains where β-phycocyanin cannot be phosphorylation can be created, thereby providing the information of the physiological function(s) of the phosphorylation of β-phycocyanin. Localisation of the phosphorylation site would be carried out in two stages. Firstly, identification of the phosphoamino acid and secondly, localisation of phosphorylation site(s).

3.5.1. Identification of the phosphoamino acid

Proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation, separated on an SDS-PAGE gel and blotted onto a PVDF membrane. The β-phycocyanin band was cut out, and the protein was subjected to acid hydrolysis (6 N HCl, 120°C for 2 hr). The hydrolysed phosphoamino acids were separated using thin layer chromatography (TLC) together with authentic phosphoamino acid standards. The amino acids on the TLC plate were visualised by staining with 0.5% ninhydrin in acetone (w/v) and incubation at 60°C for 30 min followed by autoradiography. Two radiolabelled spots on the autoradiograph of the TLC plate were found, one of which migrated with the same R_f as free phosphate and the other migrated with the same R_f as phosphoserine (Fig. 3.9). Therefore, the phosphoamino acid of β-phycocyanin was identified as phosphoserine.

Fig. 3.9. Identification of the phosphoamino acid of β-phycocyanin.

Phosphoamino acids were identified by thin layer chromatography of the products of acid hydrolysis (2 hr, 120°C) of phosphorylated β-phycocyanin. The phosphorylated amino acids were detected by autoradiography (b) of the thin layer plate (a) for 2 weeks. Authentic samples of phosphoserine, phosphothreonine and phosphotyrosine were used for identifying the phosphoamino acid in the phosphorylated β-phycocyanin by staining with 0.5% (w/v) ninhydrin in acetone after chromatography.

Abbreviations: PS, phosphoserine; PT, phosphothreonine; PTy, phosphotyrosine; Pi, free phosphate.

3.5.2. Localisation of the phosphoserine

When the sucrose gradient- and SDS-PAGE-purified, phosphorylated β-phycocyanin was digested with V8 protease, a 9 kDa phosphorylated fragment could be obtained; however, this 9 kDa fragment was too large to be sequenced through to find out which particular serine residue(s) was phosphorylated. In order to obtain a smaller digestion fragment to localise the phosphorylation site(s), double digestion of the phosphorylated β-phycocyanin with different proteases was performed as follows. The sucrose gradient- and SDS-PAGE purified, phosphorylated β-phycocyanin was digested with V8 protease for 8 hr, followed by further digestion with chymotrypsin or trypsin for 3 hr. The double digestion fragments were subjected to Tricine gel analysis and autoradiography and showed that a broad [^{32}P]-radiolabelled band ranging between 5 kDa to 7 kDa was obtained when β-phycocyanin was double digested by V8 and chymotrypsin (Fig. 3.10). A broad [^{32}P]-radiolabelled band ranging between 5 kDa to 6 kDa was also found when β-phycocyanin was double digested by V8 and trypsin (data not shown). Therefore, the double digestion fragments were not well separated by the gel-based method.

Since the double digestion fragments could not be well separated by the gel-based method, an HPLC C_8 reverse-phase column was used in an attempt to separate the digestion fragments. Proteins in the phycobiliprotein-containing fractions of the sucrose gradient was subjected to *in vitro* phosphorylation. Proteins in these fractions were then digested with trypsin and then loaded on to an HPLC C_8 reverse-phase column which was previously equilibrated with 0.1% trifluoroacetic acid (pH 2.11). A linear 0-60% acetonitrile (CH_3CN) gradient was used to elute the trypsin digestion fragments and the radioactivity (^{32}P, counts per minute, cpm) in each eluted fraction (1 ml) was measured with a scintillation counter. The elution pattern and the radioactivity showed that there were two broad radioactivity peaks (Fig. 3.11). The first broad peak turned up at retention

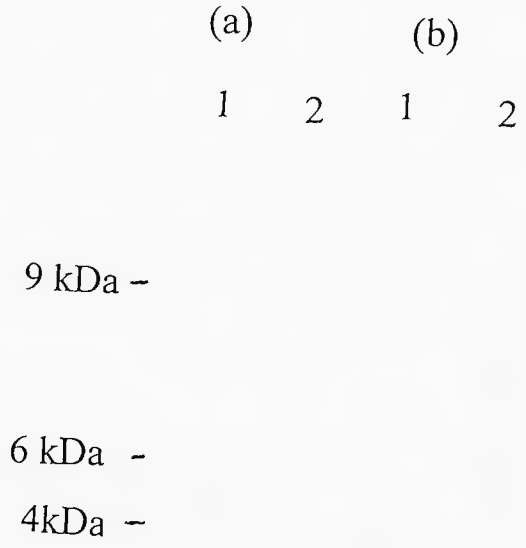

Fig. 3.10. Double digestion of sucrose gradient- and SDS-PAGE purified β-phycocyanin. Phosphorylated β-phycocyanin was digested with V8 protease for 8 hr and was further digested with chymotrypsin for 3 hr. The double digestion fragments were separated on a Tricine gel and then subjected to autoradiography. (a) Coomassie blue stained protein gel. (b) Autoradiograph. Track 1, 9 kDa fragment of V8 digested β-phycocyanin. Track 2, products of the double digested β-phycocyanin.

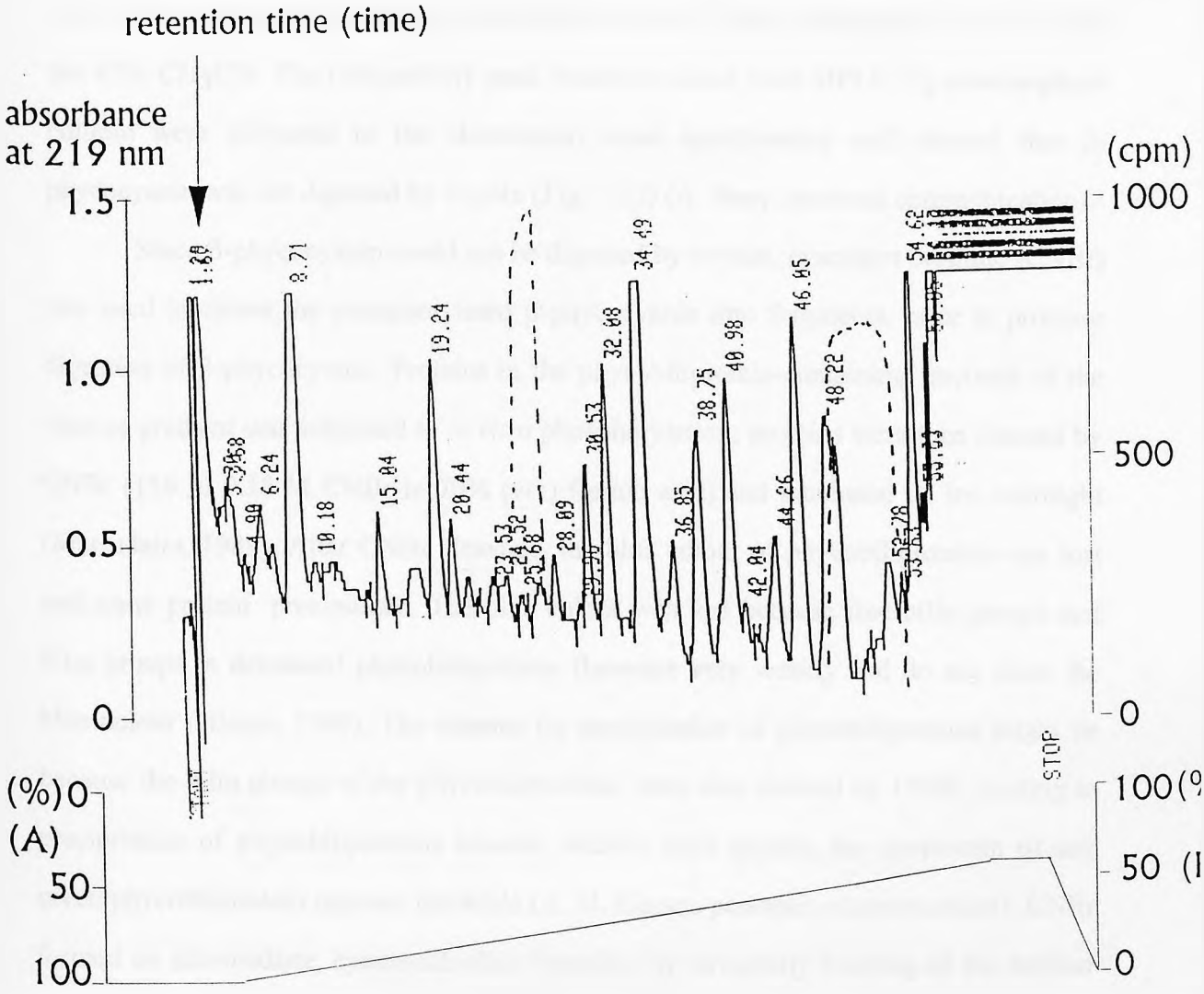

Fig. 3.11. HPLC separation of fragments of trypsin digested proteins in the phycobiliprotein-containing fractions of a sucrose gradient.

Proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation, followed by digestion with trypsin. The trypsin digested fragments were separated by an HPLC C_8 reverse-phase column using a gradient of 0-60% solvent B (CH_3CN) in 60 min where solvent A was 0.1% trifluoroacetic acid (pH 2.11). Radioactivity was determined by counting aliquots (100 µl) of fractions containing 1 ml. Solid line, absorbance at 219 nm; dashed line, radioactivity (^{32}P, counts per minute, cpm).

time between 24 and 26 min, equivalent to the 19% and the 21% CH_3CN. The second broad peak appeared at retention time between 48 and 52 min, equivalent to the 43% and the 47% CH_3CN. The radioactivity peak fractions eluted from HPLC C_8 reverse-phase column were subjected to the electrospray mass spectrometry and showed that β-phycocyanin was not digested by trypsin (Fig. 3.12) (A. Buzy, personal communication).

Since β-phycocyanin could not be digested by trypsin, cyanogen bromide (CNBr) was used to cleave the phosphorylated β-phycocyanin into fragments, prior to protease digestion of β-phycocyanin. Proteins in the phycobiliprotein-containing fractions of the sucrose gradient was subjected to *in vitro* phosphorylation; proteins were then cleaved by CNBr (150 µl 0.15 M CNBr in 70% (v/v) formic acid) and incubated on ice overnight (Matsudaira, 1989). After CNBr cleavage, the blue colour of phycobiliproteins was lost and some protein precipitated. The blue colour was lost because free bilin groups and bilin groups in denatured phycobiliproteins fluoresce very weakly and do not show the blue colour (Glazer, 1989). The reasons for precipitation of phycobiliproteins might be because the bilin groups of the phycobiliproteins were also cleaved by CNBr, leading to precipitation of phycobiliproteins because without bilin groups, the apoprotein of any given phycobiliprotein become insoluble (A. N. Glazer, personal communication). CNBr formed an intermediate, cyanosulfonium bromide, by covalently bonding to the sulphur atom in a methionyl peptide through an electron pair, thereby cleaving the protein into fragments (Cooper, 1977). We could not rule out the possibility that CNBr also form an intermediate by bonding to the sulphur atom of the three cysteine residues (α84, β84 and β155), which are covalently linked to the bilin groups, in the phycobiliproteins and thus removed the three bilin groups. Another possibility was that formic acid used in the CNBr cleavage might denature proteins and resulted in protein precipitation.

Since the particular serine which was phosphorylated was still not determined, a molecular biology would have to be employed (Chapter 5).

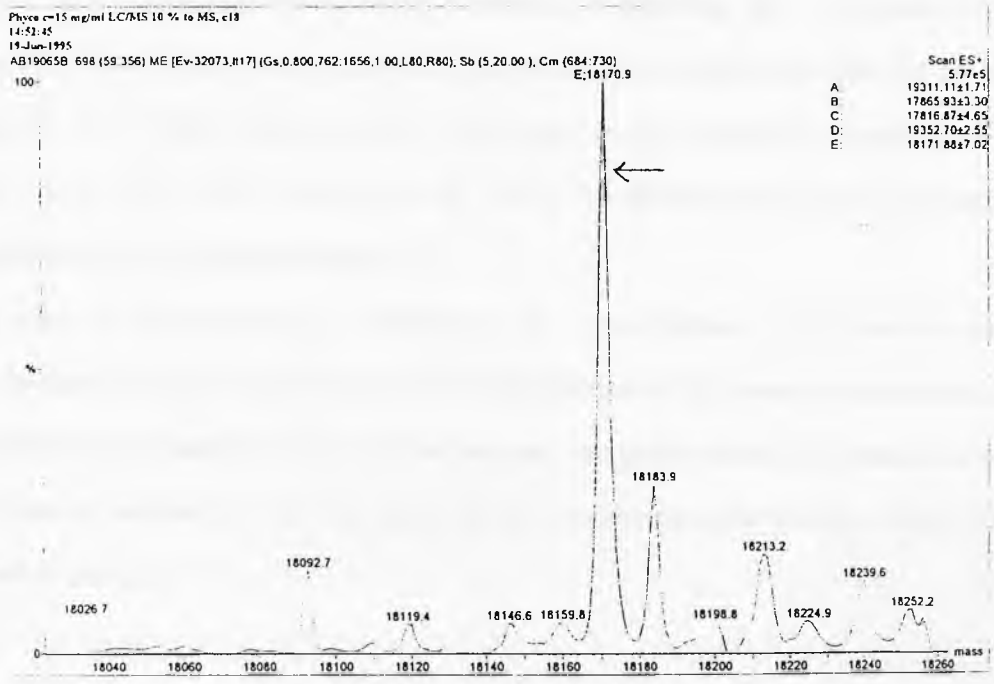

Fig. 3.12. Electrospray mass spectrometry of fragments of trypsin digested proteins in the phycobiliproteins-containing fractions of a sucrose gradient.

Proteins in the phycobiliprotein-containing fractions of a sucrose grdient were subjected to *in vitro* phosphrylation and then were digested by trypsin. Fragments of the trypsin digested proteins were separated using an HPLC C_8 reverse-phase column and then subjected electrospray mass spectrometry analysis. The arrow indicates phosphorylated β -phycocyanin (Courtesy of Dr. A. Buzy).

3.6. Phosphorylation and dephosphorylation of β-phycocyanin

One aim of this project was to establish the physiological role of protein phosphorylation. However, previous time course experiments had shown that the labelling of the β-phycocyanin (the 18 kDa protein) decreased, when the incubation time for the *in vitro* phosphorylation was increased, suggesting the existence of a phosphatase(s). Since phosphatase activities have been shown in the cell-free extracts in *Anabaena* sp. PCC 7120 (Mann *et al.*, 1991) and in the thylakoid membranes of *Synechococcus* sp. PCC 6301 (Harrison *et al.*, 1991), we therefore also tried to examine the dephosphorylation of β-phycocyanin.

In order to demonstrate the occurrence of a phosphatase, two attempts were employed to reach the goal. The first one was the dilution of the protein concentration, which is described in Section 3.7.1. and the second was pulse-chase reactions with the addition of excess unlabelled ATP, preceded by the *in vitro* phosphorylation, which will be described in Section 3.7.2.

3.6.1. The dilution experiments

If there is an inhibitor(s) of the protein kinase or a phosphatase(s), the intensity of the labelling of proteins *in vitro* should increase and/or some more proteins would be phosphorylated when the inhibitor(s) of protein kinases and the phosphatase(s) are diluted, but total protein kept constant.

The phycobiliprotein-containing fractions of the sucrose gradient were diluted by addition of different amounts of TES buffer; proteins were then phosphorylated *in vitro*, separated by SDS-PAGE analysis and subjected to autoradiography. These assays showed when the protein concentration in these fractions was diluted the intensity of radioactivity of [^{32}P] in the phosphorylated β-phycocyanin increased, and some proteins

other than β-phycocyanin became phosphorylated, especially a 19 kDa protein, suggesting that phosphatase activities or protein kinase inhibitor(s) in the phycobiliprotein-containing fractions of the sucrose density gradient (Fig. 3.13). Sometimes, a 20 kDa protein and a 56 kDa protein were also phosphorylated, although, as in the previous experiments, phosphorylation of these two proteins was variable from experiment to experiment (data not shown). However, it was still unknown whether there was an inhibitor(s) of the protein kinases or the phosphatase. In order to investigate this, a pulse-chase reaction was be used to obtain further information.

3.6.2. The existence of phosphatase activity

In order to detect phosphatase activities, pulse-chase experiments were performed by the addition of excess (1 mM, final concentration) unlabelled ATP, following the *in vitro* phosphorylation. In the event of any phosphatase activity, the [^{32}P] labelling of the phosphorylated protein would be removed by phosphatase as long as the proteins are not irreversibly phosphorylated. Although there will still be protein kinase activity in the reaction mixtures, the excess of unlabelled ATP (1000 fold of the [γ-^{32}P]ATP would minimise further incorporation of isotope.

Proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation, followed by a pulse-chase with the addition of excess unlabelled ATP and different incubation times. No labelling of β-phycocyanin could be detected after addition of excess (1 mM) cold ATP, confirming the existence of phosphatase activity (Fig. 3.14). As described earlier, a 20 kDa protein and a 56 kDa were sometimes found to be phosphorylated from experiment to experiment. *In vitro* phosphorylation of the phycobiliprotein-containing fractions, followed by a pulse-chase with the addition of excess unlabelled ATP, also indicated that the 56 kDa protein could be phosphorylated and dephosphorylated; whereas the 20 kDa protein could be

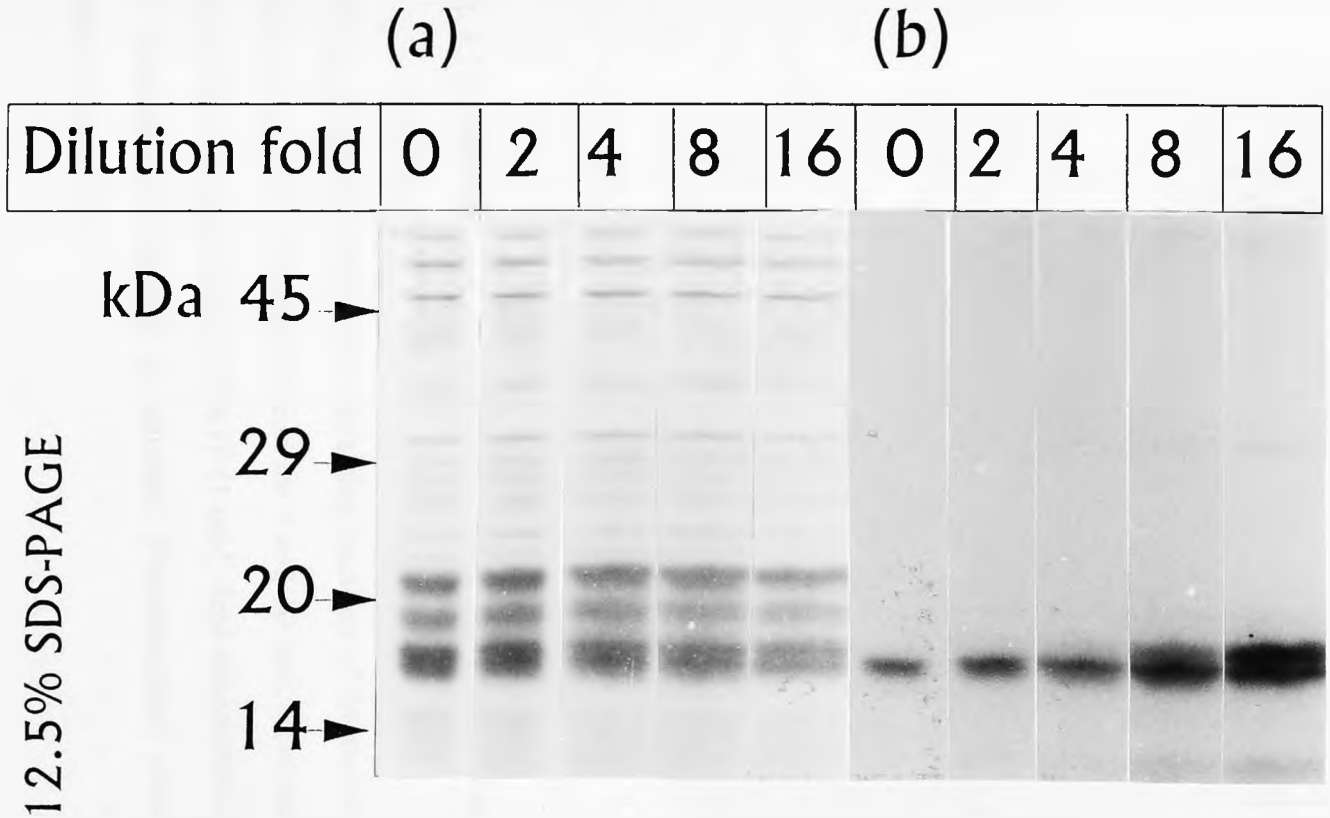

Fig. 3.13. Dilution effect on phosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose gradient.

Protein concentration in the phycobiliprotein-containing fractions of the sucrose gradient was diluted with different amounts of 20 mM TES, pH 7.5. Proteins were subjected to *in vitro* phosphorylation and were then separated by a 12.5% SDS-PAGE gel, followed autoradiography. (a) Coomassie blue stained protein gel. (b) Autoradiograph. Different dilution are as indicated. Total protein loaded per track was constant.

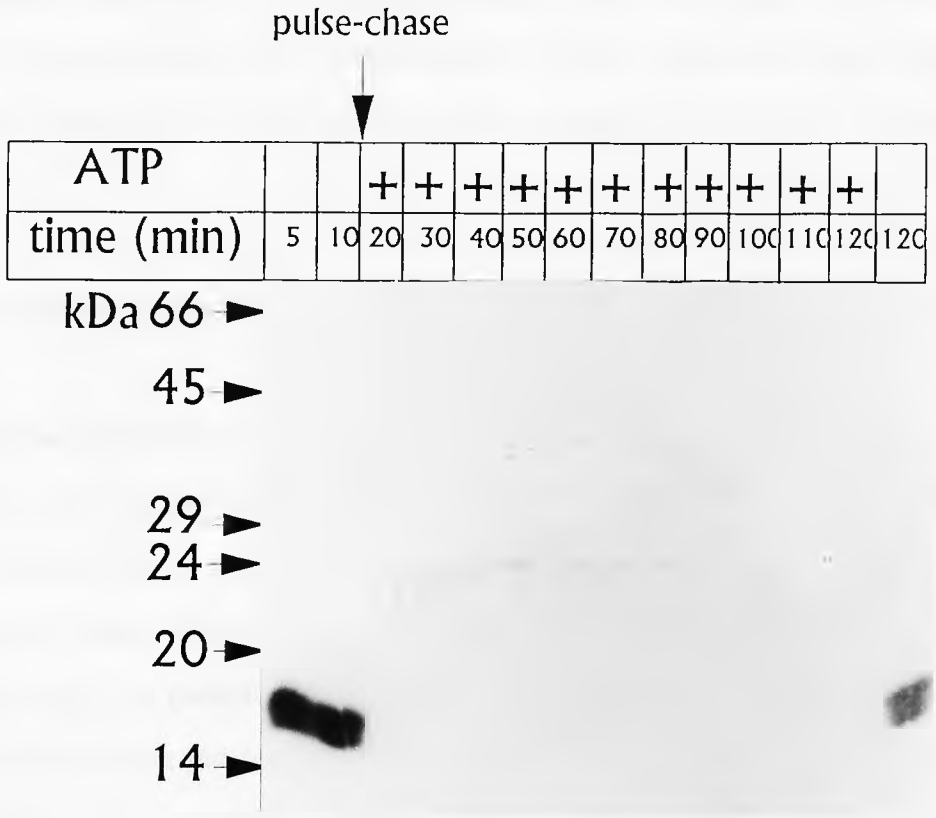

Fig. 3.14. Dephosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient.

Proteins in the phycobiliprotein-containing fractions of the sucrose density gradient were subjected to *in vitro* phosphorylation for 5 and 10 min, followed by a pulse-chase with the addition of excess unlabelled ATP (1 mM, final concentration) and further incubated for different time intervals as indicated. Phosphorylated proteins are shown on the autoradiograph.

phosphorylated, but could not be dephosphorylated (data not shown). Therefore, the existence of a phosphatase(s) was demonstrated with both the protein kinase and the phosphatase co-occurring in the phycobiliprotein-containing fractions of the sucrose gradient.

3.7. Factors affecting phosphorylation and dephosphorylation of β-phycocyanin

Phosphorylation and dephosphorylation of proteins have been shown to be inhibited by some inhibitors of protein kinases and phosphatases in *Synechococcus* sp. PCC 6301 (Harrison *et al.*, 1991). In our experiments, β-phycocyanin was phosphorylated by a protein kinase (hereafter referred to as the β-phycocyanin kinase) and dephosphorylated by a phosphatase; accordingly, we decided to attempt to investigate whether phosphorylation and dephosphorylation of β-phycocyanin could also be inhibited by inhibitors of protein kinases and phosphatases. FSBA (5'-*p*-flourosulfonylbenzoyladenosine), a commonly used protein kinase inhibitor, microcystin-LR, a eukaryotic protein phosphatase type 1 and type 2A inhibitor, NaF, a commonly used, non-specific protein phosphatase inhibitor and EDTA, a divalent cation chelator were all investigated.

The phycobiliprotein-containing fractions of the sucrose gradient were preincubated for 5 min in the presence of FSBA, EDTA and microcystin-LR and proteins in these fractions were then subjected to *in vitro* phosphorylation, followed by SDS-PAGE analysis and autoradiography. These assays showed that phosphorylation of β-phycocyanin was inhibited by EDTA; however, FSBA and microcystin-LR did not affect the phosphorylation of β-phycocyanin. This suggested that Mg^{2+} or another divalent cation was required for the β-phycocyanin kinase activity (Fig. 3.15). Proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation. FSBA, EDTA, microcystin-LR and NaF were then individually added

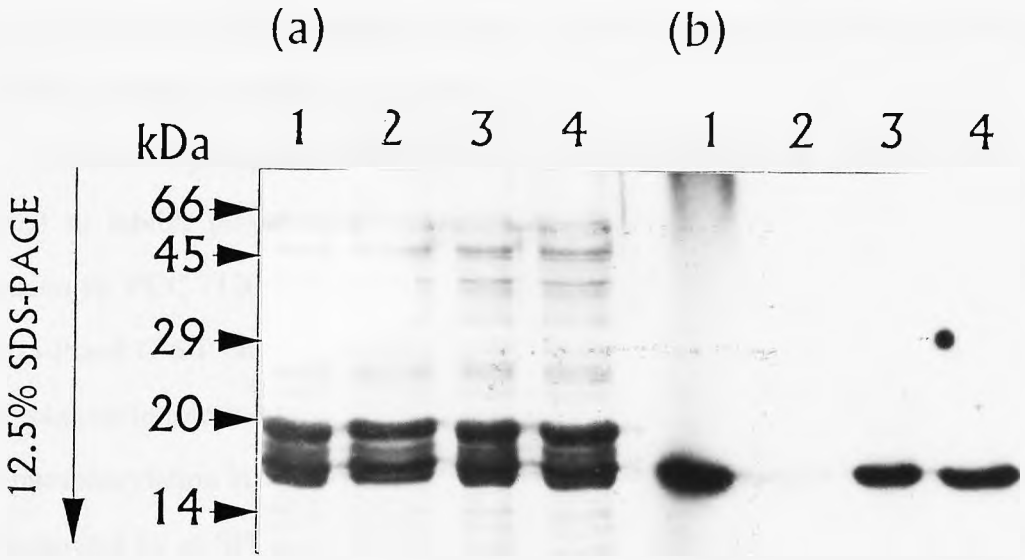

Fig. 3.15. Inhibition of phosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient.

Proteins in the phycobiliprotein-containing fractions of the sucrose density gradient were incubated with EDTA, FSBA, microcystin-LR or the phosphorylation reaction buffer (20 mM TES buffer, pH 7.5) for 10 min, followed by *in vitro* phosphorylation. Proteins were separated by 12.5% SDS-PAGE gel and subjected to autoradiography. (a) Coomassie stained protein gel. (b) Autoradiograph. Different chemicals added in each track are as follows: 1, 20 mM TES buffer, pH 7.5; 2, EDTA; 3, FSBA; 4, microcystin-LR.

Abbreviation: FSBA, 5'-p-fluorosulfonylbenzoyladenosine.

into these phosphorylation reactions mixtures, followed by a pulse-chase with the addition of excess unlabelled ATP (1 mM) and proteins were then subjected to SDS-PAGE analysis and autoradiography. These assays showed that dephosphorylation of β-phycocyanin was inhibited by EDTA, suggesting that Mg^{2+} or another divalent cation was also required for the phosphatase activity; FSBA, microcystin-LR and NaF did not inhibit the dephosphorylation reaction (Fig. 3.16).

Ribulose-5-phosphate (Ru-5-P) and glucose-6-phosphate (G-6-P) have been reported to inhibit phosphorylation of a 56 kDa protein in the cell-free extracts of *Anabaena* sp. PCC 7120 by different mechanisms (Mann *et al.*, 1991). Therefore, effects of Ru-5-P and G-6-P on phosphorylation of β-phycocyanin were also examined. Proteins in the phycobiliprotein-containing fractions of the sucrose gradient were subjected to *in vitro* phosphorylation in the presence or in the absence of Ru-5-P or G-6-P; proteins were then separated by an SDS-PAGE gel, followed by autoradiography. These assays showed that both Ru-5-p and G-6-P did not affect the phosphorylation of β-phycocyanin (Fig. 3.17). In contrast, phosphorylation of the 56 kDa protein was inhibited by both Ru-5-P and G-6-P, which was in accordance with that found in *Anabaena* sp. PCC 7120 (Mann *et al.*, 1991).

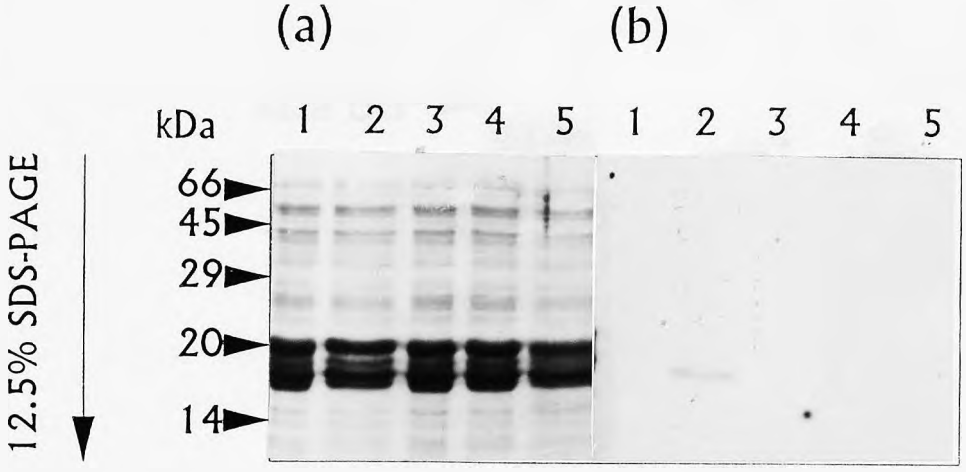

Fig. 3.16. Inhibition of dephosphorylation of phosphorylated proteins in the phycobiliprotein-containing fractions of a sucrose density gradient.

Proteins in the phycobiliprotein-containing fractions of the sucrose density gradient were subjected to *in vitro* phosphorylation for 10 min. FSBA, EDTA, microcystin-LR, NaF and the phosphorylation/dephosphorylation reaction buffer (20 mM TES, pH 7.5) were then individually added to the samples and further incubation for 10 min, followed by pulse-chase by addition of excess unlabelled ATP (1 mM) and incubated for another 10 min. Proteins were separated by 12.5% SDS-PAGE gel and subjected to autoradiography. (a) Coomassie blue stained protein gel. (b) autoradiograph. Track 1, 20 mM TES buffer, pH 7.5; track 2, EDTA; track 3, FSBA; track 4, microcystin-LR; track 5, NaF.

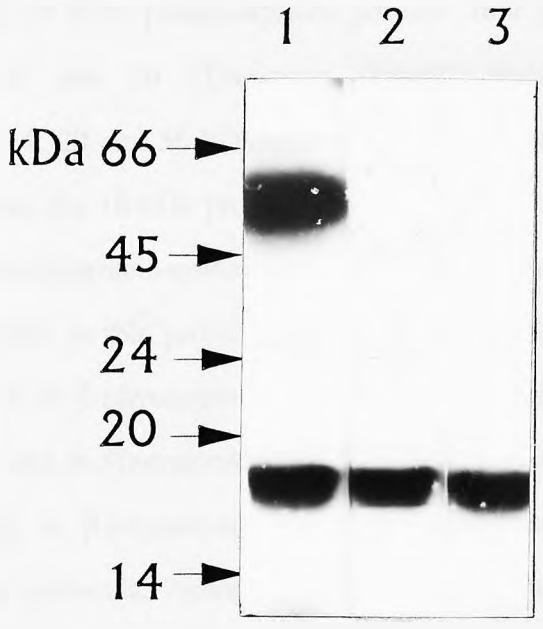

Fig. 3.17. Effects of ribulose-5-phosphate (Ru-5-P) and glucose-6-phosphate (G-6-P) on phosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient.

Phycobiliprotein-containing fractions of a sucrose density gradient were incubated in Ru-5-P or G-6-P or phosphorylation/dephosphorylation reaction buffer (20 mM TES buffer, pH 7.5) for 30 min and proteins were subjected to *in vitro* phosphorylation. Proteins were then separated by a 12.5% SDS-PAGE gel, followed by autoradiography. Each track of the autoradiograph is as follows; (1) 20 mM TES buffer, pH 7.5; (2) incubation with Ru-5-P; (3) incubation with G-6-P.

3.8. Discussion

Some proteins occurring in the thylakoid membranes and the phycobiliprotein-containing fractions resolved using a sucrose density gradient were found to be phosphorylated *in vitro*, confirming the existence of protein kinase activities in the cell-free extracts, which was consistent with the results of other workers (Mann *et al.*, 1991; Hagemann *et al.*, 1993). Of these phosphorylated proteins, four proteins with molecular weight of 14, 18, 20 and 56 kDa were strongly phosphorylated. However, phosphorylation of the 14, 20 and 56 kDa proteins were not consistent. Of the several phosphorylatable proteins, the 18 kDa protein was found to occur in both the thylakoid membranes and the phycobiliprotein-containing fractions of the sucrose gradient.

The 18 kDa protein in this project was tentatively identified as β-phycocyanin. The phosphoamino acid of β-phycocyanin was identified as phosphoserine, which is inconsistent with that found in *Synechococcus* sp. PCC 6301 (Harrison, 1990). However, which serine residue(s) in β-phycocyanin was phosphorylated still has yet to be determined. The use of molecular biology to create site-directed mutations where β-phycocyanin cannot be phosphorylated would be another approach to be employed for localisation of the phosphorylation site.

That phycobiliproteins were not digested by trypsin which was also reported for *Synechocystis* sp. PCC 6308 (Duke *et al.*, 1989). It is still not well understood why phycobiliproteins cannot be digested by trypsin; however, the protein structure of phycobiliproteins might be the reason for difficulty in digesting the phycobiliproteins by a protease(s) because the compact waterwheel structure of the phycobiliproteins (Schirmer *et al.*, 1985; 1986) may make proteases inaccessible to the digestion site in the (αβ) trimer of phycobiliproteins.

Both the dilution experiments and *in vitro* phosphorylation of β-phycocyanin, followed by a pulse-chase with addition of excess unlabelled ATP demonstrated the

existence of a phosphatase. Both phosphorylation and dephosphorylation of β-phycocyanin were insensitive to classical inhibitors of protein kinases and phosphatases, but were inhibited by EDTA, suggesting that phosphorylation and dephosphorylation of β -phycocyanin required Mg^{2+} or another divalent cation. That phosphorylation of β-phycocyanin was insensitive to FSBA and that dephosphorylation of β-phycocyanin was insensitive to NaF were in accordance with that found in *Synechococcus* sp. PCC 6301 (Harrison *et al.*, 1991). In our experiments, not only was β-phycocyan found to be phosphorylated, but some other proteins were also found to be phosphorylated, especially a 14 kDa, a 20 kDa and a 56 kDa proteins. However, these three proteins were not consistently phosphorylated *in vitro*. That phosphorylation of the 14, 20 and 56 kDa proteins were inconsistent might result from the outgrowth of cells (Köhler and Antranikian, 1989), stress (Balodimos *et al.*, 1990) or the cellular nutrition status (Forchhammer and Tandeau de Marsac, 1994). Nevertheless which factor(s) that resulted in the inconsistent phosphorylation of these three proteins still need to be determined. Sometimes, we also found that phosphorylation of β-phycocyanin and phosphorylation of the 14 kDa protein occurred as a doublet. The doublet of the phosphorylated proteins could be due to different numbers of amino acid residue in the same protein molecule that was phosphorylated, which affected their mobility when the proteins were subjected to SDS-PAGE analysis. That different numbers of amino acid residue of the protein molecule that was phosphorylted might be because of the nutrition status of during the course of cell culture (Forchhammer and Tandeau de Marsac, 1994). However, the physiological function of different numbers of phosphoamino acids in the 14 kDa and β-phycocyanin still needs to be determined.

Chapter 4

Partial Purification of β-phycocyanin Kinase from *Synechocystis* sp. PCC 6803

4.1. Introduction

It is well established that proteins in the light-harvesting chlorophyll a/b protein complexes (LHC) are phosphorylated by protein kinases (for review see Bennett, 1991). The existence of protein kinase and phosphatase activities was also demonstrated (Bennett, 1977; Bennett, 1980). Phosphorylation of thylakoid membrane proteins is under redox control (Silverstein *et al.*, 1993a); however, protein phosphatase reactions have been found to be redox-independent (Silverstein *et al.*, 1993b). Several lines of evidence support the existence of multiple thylakoid membrane protein kinases (for review see Allen, 1992) and three protein kinases have been purified from spinach thylakoids (Lin *et al.*, 1982; Lucero *et al.*, 1982; Coughlan and Hind, 1986a).

The existence of protein kinase and phosphatase activities has also been demonstrated in cyanobacteria (Schuster *et al.*, 1984; Sanders and Allen, 1987; Harrison *et al.*, 1991; Mann *et al.*, 1991; Hagemann *et al.*, 1993). Nevertheless, neither a protein kinase nor a phosphatase has been purified from cyanobacteria so far.

Previous experiments (Chapter 3) had shown that β-phycocyanin was phosphorylated, and that a serine residue(s) was modified. The existence of a protein kinase which phosphorylated β-phycocyanin was demonstrated; additionally, pulse-chase reactions confirmed the existence of a phosphatase which dephosphorylated β-phycocyanin. Both phosphorylation and dephosphorylation of β-phycocyanin were occurring simultaneously and were insensitive to standard inhibitors of protein kinases and phosphatases. Attempts at purification of the kinase phosphorylating β-phycocyanin are therefore described in this chapter. There are two approaches which can be employed to isolate a protein of interest. Firstly, the use of biochemical methods to purify the protein and secondly, the use of the techniques of molecular biology to clone, sequence and express the gene. The use of the biochemical approach to purify this kinase were thought to be more suitable in the first instance.

4.2. Phycobiliproteins are acidic proteins

Phycobiliproteins are richer in acidic rather than basic amino acids and that the phycobiliproteins are acidic proteins has been found for several strains of cyanobacteria (Berns *et al*, 1964; Cope *et al*, 1967; Raftery and O'hEocha, 1965). Indeed, all the phycobiliproteins have pIs ranging between pH 4-5.5 (for review see Cohen-Bazire and Bryant, 1982). C-phycocyanin, C-allophycocyanin and C-phycoerythrin from several strains of cyanobacteria have been purified using anion exchange chromatography (Glazer 1988b).

The phycobiliprotein-containing fractions of the sucrose gradient were collected and proteins in these fractions were subjected to isoelectric focusing, followed by the 12.5% SDS-PAGE analysis. This 2-D gel analysis showed that the pI of β-phycocyanin of *Synechocystis* sp. PCC 6803 was about 4.0 (Fig. 4.1). β-phycocyanin on the 2-D gel could be identified by either *in vitro* phosphorylation of, followed by autoradiography or by the measurement of the R_f relative to the C-phycocyanin standard (data not shown).

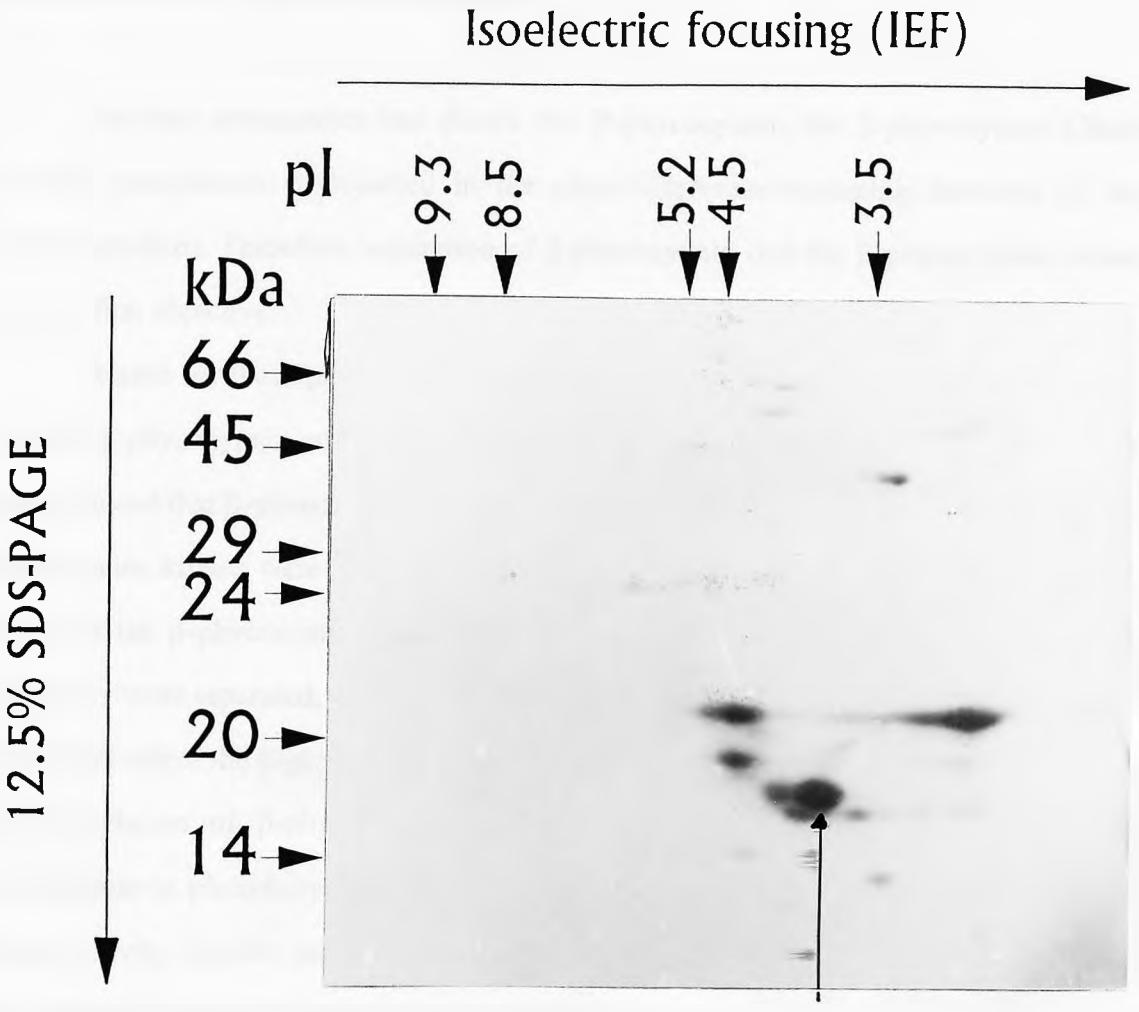

Fig. 4.1. Two-dimensional gel electrophoresis of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient.

Proteins in the phycobiliprotein-containing fractions of the sucrose density gradient were separated by isoelectric focusing and then further separated by 12.5% SDS-PAGE gel, which was stained by Coomassie blue. The arrow indicates β-phycocyanin.

4.3. Separation of β-phycocyanin kinase

Previous experiments had shown that β-phycocyanin, the β-phycocyanin kinase and the phosphatase co-occurred in the phycobiliprotein-containing fractions of the sucrose gradient. Therefore, separation of β-phycocyanin and the β-phycocyanin kinase was the first objective.

Assays for the β-phycocyanin kinase activity were be used as the indicator to see whether β-phycocyanin and the β-phycocyanin kinase were separated. If *in vitro* kinase assay showed that β-phycocyanin was not phosphorylated, then β-phycocyanin and the β-phycocyanin kinase were assumed to be separated into different fractions. Therefore, assay for the β-phycocyanin kinase activity was performed after each separation step. Once they were separated, if the β-phycocyanin-containing fraction and the other protein fraction(s) where the β-phycocyanin kinase maybe exists are mixed, followed by *in vitro* phosphorylation of β-phycocyanin and if *in vitro* phosphorylation shows that β-phycocyanin is phosphorylated, it is assumed the other protein fraction(s) contains the kinase activity, thereby purifying the β-phycocyanin kinase. Therefore, assay for the β-phycocyanin kinase will be carried out after any mixing step.

4.3.1. Anion exchange chromatography

Nothing about the β-phycocyanin kinase except its kinase activity was known; in contrast, β-phycocyanin both in terms of the protein structure and the gene structure is well understood. Since β-phycocyanin is an acidic protein, anion exchange chromatography was initially used to try to separate β-phycocyanin and the β-phycocyanin kinase. The phycobiliprotein-containing fractions of the sucrose density gradient were loaded onto a Mono Q column, which had been previously equilibrated with 20 mM Tris.HCl buffer, pH 7.5 and a linear 0-1 M KCl gradient was used to elute

proteins. The elution pattern showed that four major protein peaks were eluted from the Mono Q column between the concentrations of 0.1 to 0.4 M KCl; some minor peaks with small amounts of protein were eluted between the concentrations of 0.4 to 0.9 M KCl (Fig. 4.2). Fractions eluted from the Mono Q column in the concentration ranging from 0.2 to 0.4 M KCl were blue. SDS-PAGE analysis together with UV light illumination showed that these fractions were mainly composed of phycobiliproteins (data not shown).

Proteins in each fraction (1 ml) eluted from the Mono Q column were subjected to *in vitro* phosphorylation, followed by SDS-PAGE analysis and autoradiography. these assays showed that β-phycocyanin occurred in the fractions eluted at the concentrations of 0.30 to 0.35 M KCl and was strongly phosphorylated, indicating that both β-phycocyanin and β-phycocyanin kinase co-occurred in the same fractions (Fig. 4.3). Previous experiments had shown that in the phycobiliprotein-containing fractions of the sucrose gradient, a 20 kDa and a 56 kDa protein were inconsistently phosphorylated from experiment to experiment. However, in the β-phycocyanin-containing fractions eluted from the Mono Q column, only β-phycocyanin was found to be phosphorylated.

Previous experiments had shown that β-phycocyanin, the β-phycocyanin kinase and the phosphatase co-occurred in the phycobiliprotein-containing fractions of the sucrose density gradient. Therefore, besides assaying the β-phycocyanin kinase, we also tried to detect the phosphatase. The β-phycocyanin-containing fractions eluted from the Mono Q column at 0.30 and 0.35 M KCl were collected and pooled together. Proteins in these fractions were subjected to *in vitro* phosphorylation, followed by a pulse-chase with the addition of excess of unlabelled ATP. Proteins were then subjected to SDS-PAGE analysis and autoradiography. No labelling of β-phycocyanin could be detected after addition of excess cold ATP, suggesting that the phosphatase activity also occurred in the β-phycocyanin-containing fractions eluted from the Mono Q column between 0.30 to 0.35 M KCl (Fig. 4.4).

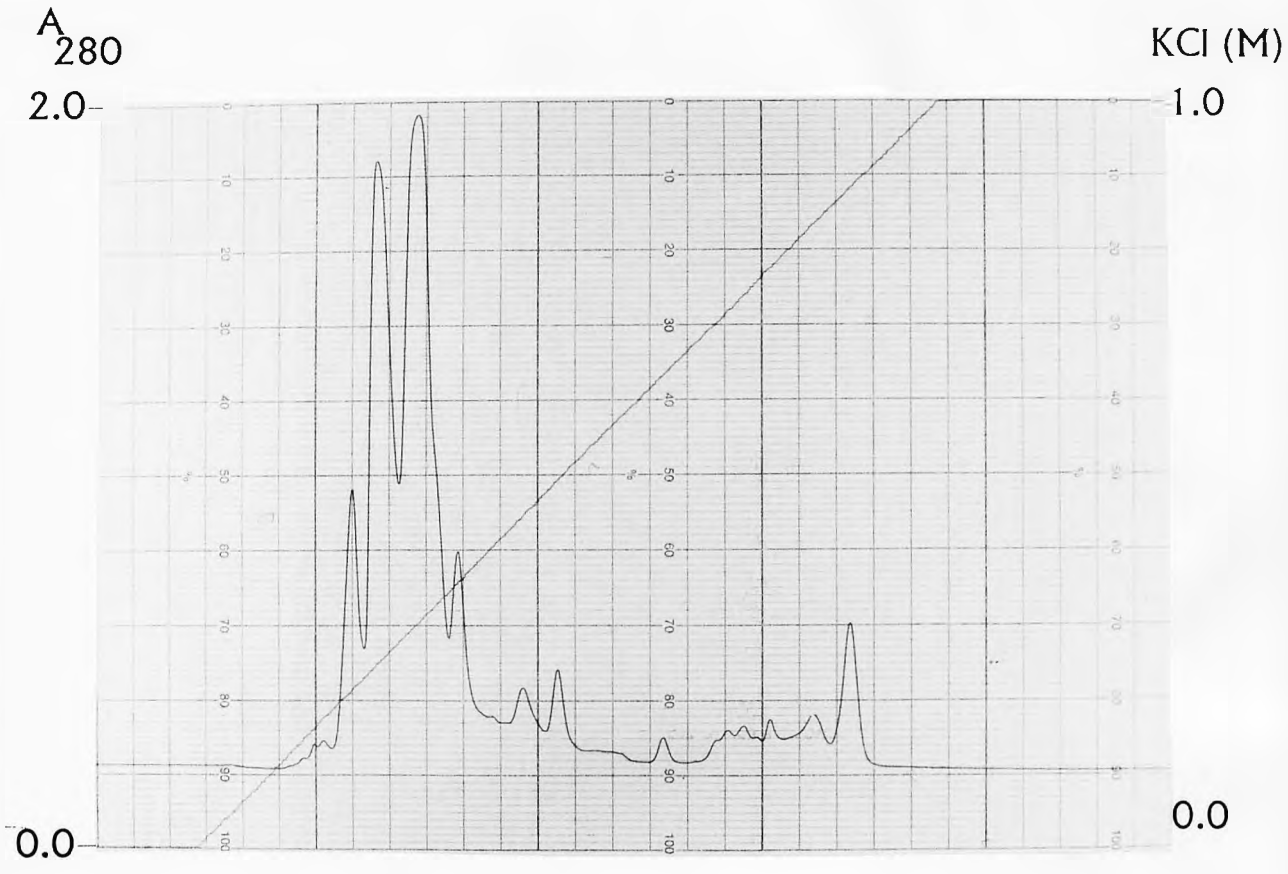

Fig. 4.2. Separation of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient by anion exchange chromatography on an FPLC Mono Q column.

The phycobiliprotein-containing fractions of the sucrose density gradient were subjected to anion exchange chromatography on an FPLC Mono Q column. Eluent: 20 mM Tris-HCl, pH 7.5. A linear 0-1 M KCl gradient was used to elute proteins. Flow rate: 1 ml/min.

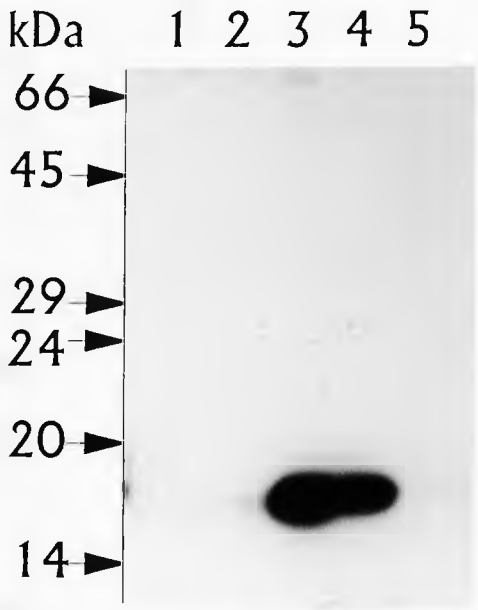

Fig. 4.3. Protein kinase activity assay of fractions eluted from an FPLC Mono Q column. The phycobiliprotein-containing fractions of the sucrose density gradient were pooled and loaded on an FPLC Mono Q column. Each fraction eluted from the Mono Q column was subjected to *in vitro* phosphorylation. Proteins were separated by 12.5% SDS-PAGE gel and subjected to autoradiography. Concentrations of the linear 0-1 M KCl gradient for eluting proteins from the Mono Q are indicated in each track; (1) 0.2 M KCl; (2) 0.25 M KCl; (3) 0.3 M KCl; (4) 0.35 M KCl; (5) 0.4 M KCl.

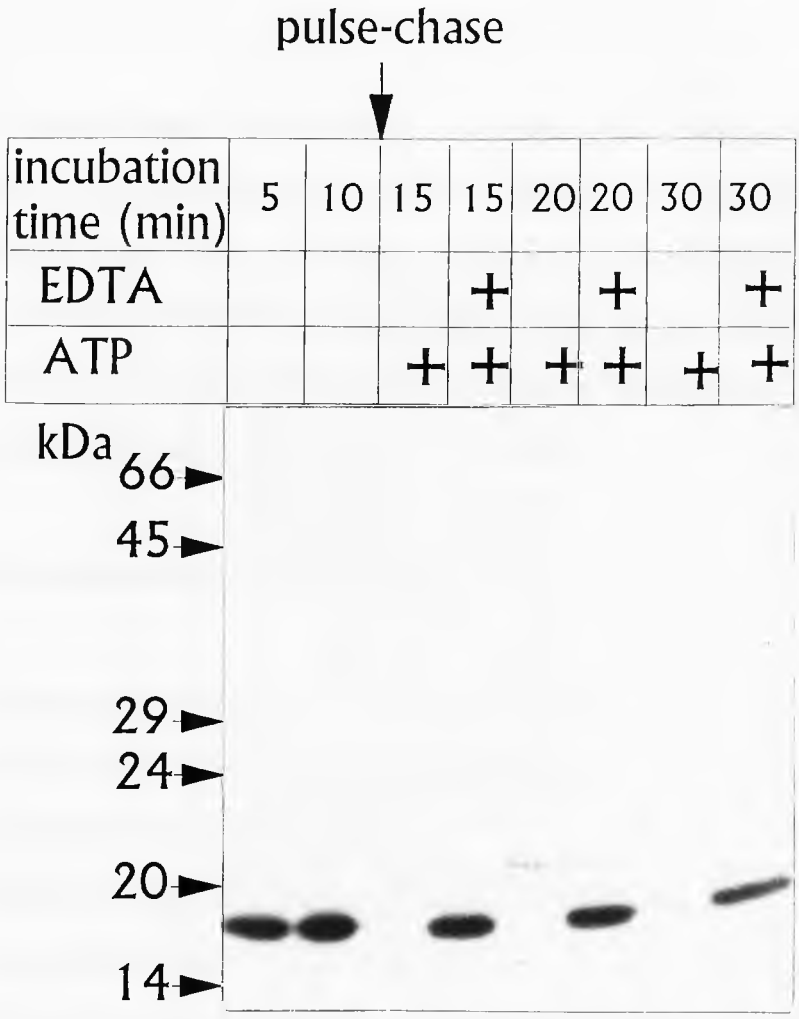

Fig. 4.4. Autoradiograph of the *in vitro* phosphorylation and dephosphorylation of proteins in the fractions eluted from an FPLC between 0.3 and 0.35 M KCl Mono Q column.

Fractions eluted from the FPLC Mono Q column at 0.3-0.35 MCl were collected, assayed by *in vitro* phosphorylation and then incubated with (+) or without (-) EDTA, followed by pulse-chase with the addition of excess unlabelled ATP (+) or without addition of unlabelled AT (-)P. Proteins were separated by a 12.5% SDS-PAGE gel and subjected to autoradiography. Different incubation times, pulse-chase by adding excess unlabelled ATP [1 M] and the addition of EDTA are as indicated.

EDTA has previously been found to inhibit the phosphorylation and dephosphorylation of β-phycocyanin. Therefore, proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column were subjected to *in vitro* phosphorylation, and then EDTA (10 mM) was added in these fractions and incubated for 10 min, followed by a pulse chase with the addition of excess of unlabelled ATP. We also found that dephosphorylation of β-phycocyanin was inhibited (Fig. 4.4).

4.3.2. Gel filtration chromatography

Since β-phycocyanin, β-phycocyanin kinase and the phosphatase co-occurred in the phycobiliprotein-containing fractions and ion exchange chromatography had failed to separate them, gel filtration chromatography on an FPLC Superose 12 column (hereafter referred to as Superose 12 column) was used to attempt to separate them.

The β-phycocyanin-containing fractions eluted from the Mono Q column were collected, concentrated and then loaded onto a Superose 12 column, which had been previous equilibrated with 20 mM Tris.HCl buffer pH 7.5 containing 0.1 M KCl. The addition of 0.1 M KCl to the buffer was to safeguard against possible ionic interactions between the proteins and the gel matrix. The elution pattern showed that two protein peaks were eluted from the Superose 12 column (Fig. 4.5). Calibration of the Superose 12 column with molecular weight markers showed that the first protein peak had an apparent molecular mass of about 120 kDa; the molecular mass of the second protein peak was about 70 kDa. The first protein peak was blue and therefore contained phycobiliproteins.

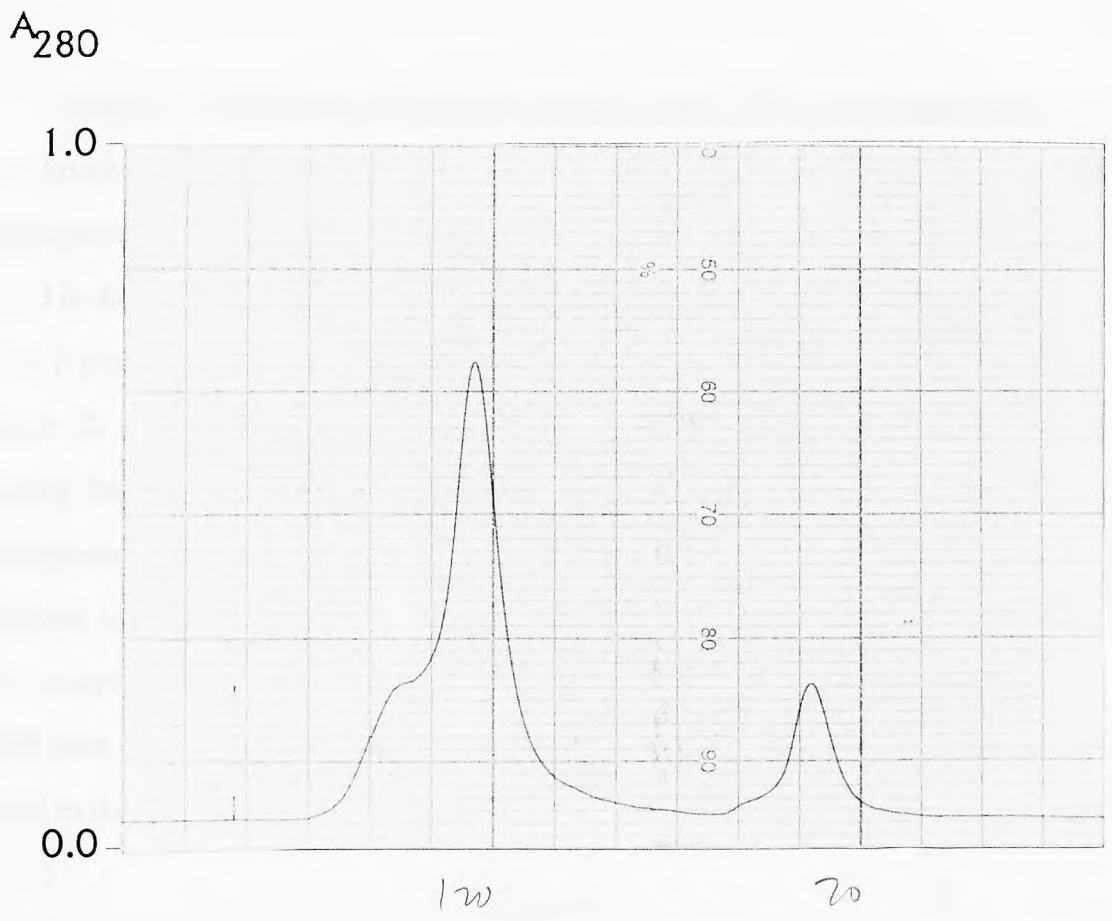

Fig. 4.5. Separation of proteins in fractions eluted from the Mono Q column between 0.3 and 0.35 M KCl by an FPLC Superose 12 column.

Phycobiliprotein-containing fractions eluted from an FPLC Mono Q column were pooled together, concentrated and loaded onto an FPLC Superose 12 column. Eluent: 20 mM Tris-HCl, pH 7.5 containing 0.1 M KCl. Flow rate: 0.3 ml/min.

Proteins in each fraction of the two elution protein peaks were subjected to *in vitro* phosphorylation and then separated on an SDS-PAGE gel, followed by autoradiography. The *in vitro* phosphorylation assays showed that β-phycocyanin occurred in the first peak and was strongly phosphorylated, indicating that β-phycocyanin and the β-phycocyanin kinase still co-occurred in the same fractions eluted from the Superose 12 column (Fig. 4.6). To search for the phosphatase, the β-phycocyanin-containing fractions eluted from the Superose 12 column were collected and proteins were subjected to *in vitro* phosphorylation by incubation with [γ-^{32}P]ATP, followed by pulse-chase with an excess of unlabelled ATP; proteins were then subjected to SDS-PAGE analysis and autoradiography. No radiolabelling of β-phycocyanin could be detected after the addition of excess cold ATP, suggesting the phosphatase activity also occurred in the β-phycocyanin-containing fractions eluted from the Superose 12 column (Fig. 4.7).

After sucrose density gradient, ion exchange chromatography and gel filtration chromatography, β-phycocyanin and β-phycocyanin kinase and the phosphatase were not resolved. It could be that β-phycocyanin, the β-phycocyanin kinase and the phosphatase were located in a macromolecular complex or that β-phycocyanin might be subjected to autophosphorylation and autodephosphorylation. However, the possibility that β-phycocyanin autophosphorylated itself is not supported because (1) there is no the -R-X-(X)-S/T-X_3-S/T- consensus sequence motif for an autophosphorylation-dependent protein serine/threonine kinase (Yang *et al.*, 1994) in the β-phycocyanin amino acid sequences and (2) β-phycocyanin in the isolated phycobilisomes did not autophosphorylate (see below, Section 4.5). Therefore, β-phycocyanin, the β-phycocyanin kinase and the phosphatase appear to occur as a macromolecular complex (hereafter referred to as the β-phycocyanin complex).

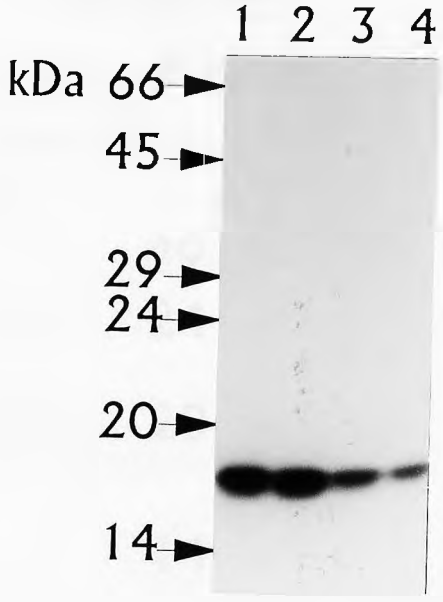

Fig. 4.6. Autoradiograph of the *in vitro* phosphorylation of proteins in the fractions eluted from the Superose 12 column.

Proteins in each fraction eluted from the FPLC Superose 12 column were subjected to *in vitro* phosphorylation. Proteins were separated by a 12.5% SDS-PAGE gel and subjected to autoradiography. The 4 fractions containing phosphorylated β-phycocyanin were located in the first peak eluted from the FPLC Superose 12 column. Tracks 1-4 represent the 4 fractions corresponding to the first peak eluted from the Superose 12 column.

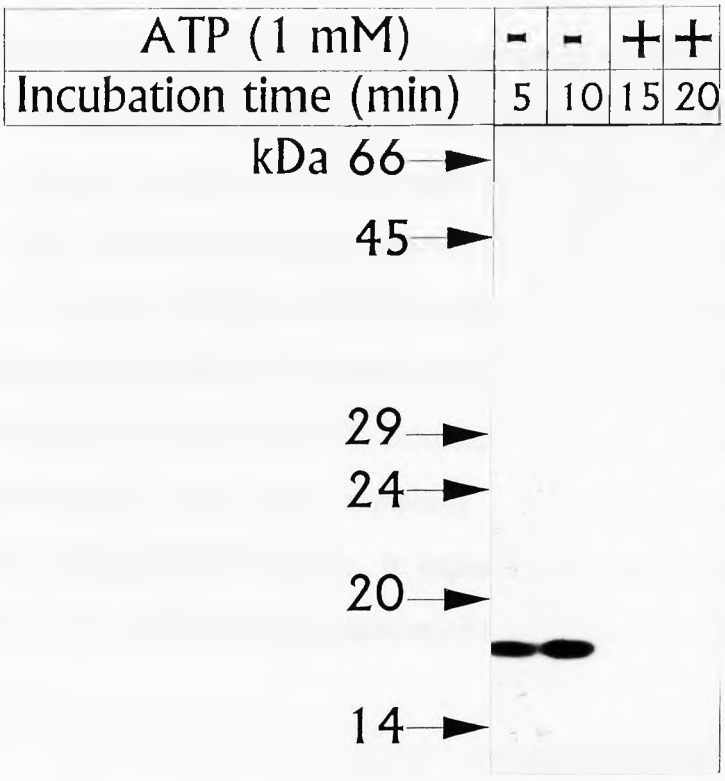

Fig. 4.7. In vitro protein kinase and phosphatase assay of proteins in the fractions of the first protein peak eluted from the FPLC Superose 12 column.

Fractions of the first protein peak eluted from the FPLC Superose 12 column were collected, proteins were subjected to *in vitro* phosphorylation, followed by pulse-chase by addition of excess unlabelled ATP. Proteins were then separated by 12.5% SDS-PAGE gel and subjected to autoradiography. Different incubation times and pulse-chase by addition of unlabelled ATP are as indicated.

4.3.3. Hydrophobic interaction chromatography (HIC)

Since β-phycocyanin and β-phycocyanin kinase could not be separated by Mono Q and Superose 12 columns, hydrophobic interaction chromatography (HIC) was used to try to separate the β-phycocyanin kinase. In order to increase the binding ability of proteins to the HIC column, ammonium sulphate is normally used to increase the hydrophobicity of proteins (Cooper, 1977). However, at a concentration of 1.8 M $(NH_4)_2SO_4$ (equivalent to about 35% saturation), neither β-phycocyanin nor β-phycocyanin kinase could bind to the HIC column; at a concentration of 2.0 M of $(NH_4)_2SO_4$ (equivalent to about 45% saturation), both β-phycocyanin and the β-phycocyanin kinase precipitated. Therefore, it appeared that hydrophobic interaction chromatography was not suitable for the separation of β-phycocyanin and β-phycocyanin kinase.

4.3.4. Phosphorylation of histone and casein

5 different types of histone and casein have been used for assay of protein kinase activity (Guo *et al.*, 1993) and histone H3 has been reported to have been used as a ligand for affinity chromatography for purifying a protein kinase form spinach thylakoid membranes (Coughlan and Hind, 1986a). Therefore, both casein and histone were tested as the substrates for the β-phycocyanin kinase. However, after *in vitro* phosphorylation assay, neither casein nor histone could be phosphorylated (data not shown), implying that β-phycocyanin kinase was specific for β-phycocyanin.

4.4. Disruption of the β-phycocyanin kinase/phosphatase complex

Previous experiments had shown that β-phycocyanin, β-phycocyanin kinase and the phosphatase occurred in what might be macromolecular complex (the "β-phycocyanin complex"). Disruption of the β-phycocyanin complex was a necessary step then for separation of β-phycocyanin and the β-phycocyanin kinase, and the phosphatase. Denaturants, such as urea and guanidinium hydrochloride denature proteins and macromolecular complexes by destroying the non-covalent interactions (Dill and Scholtz, 1991; Scholtz *et al.*, 1995); therefore these denaturants could potentially be used to denature the β-phycocyanin complex, thereby permitting separation of β-phycocyanin, the β-phycocyanin kinase and phosphatase either by ion exchange chromatography or by gel filtration chromatography.

4.4.1. The β-phycocyanin complex in the presence of urea

Different amounts of 10 M urea (in 20 mM TES buffer, pH 7.5) were added to the pooled phycobiliprotein-containing fractions of the sucrose gradient to give final concentrations of 1 to 8 M urea and the samples were incubated at 20°C for 10 min Proteins in these fractions containing different concentrations of urea were subjected to *in vitro* phosphorylation, separated on an SDS-PAGE gel and subjected to autoradiography. These assays showed that in concentrations of 1 and 5 M urea, β-phycocyanin was still phosphorylated (Fig. 4.8). The pulse-chase experiments with the addition of excess ATP showed that β-phycocyanin was also dephosphorylated in concentrations of 1 and 5 M urea (data not shown). When the urea concentration was more than 6 M, no phosphorylation occurred, implying that either β-phycocyanin or the β-phycocyanin kinase was denatured. It was also noticeable that in concentrations of 1 and 2 M urea, a 20 kDa protein was also phosphorylated; when the urea concentration was higher than 3

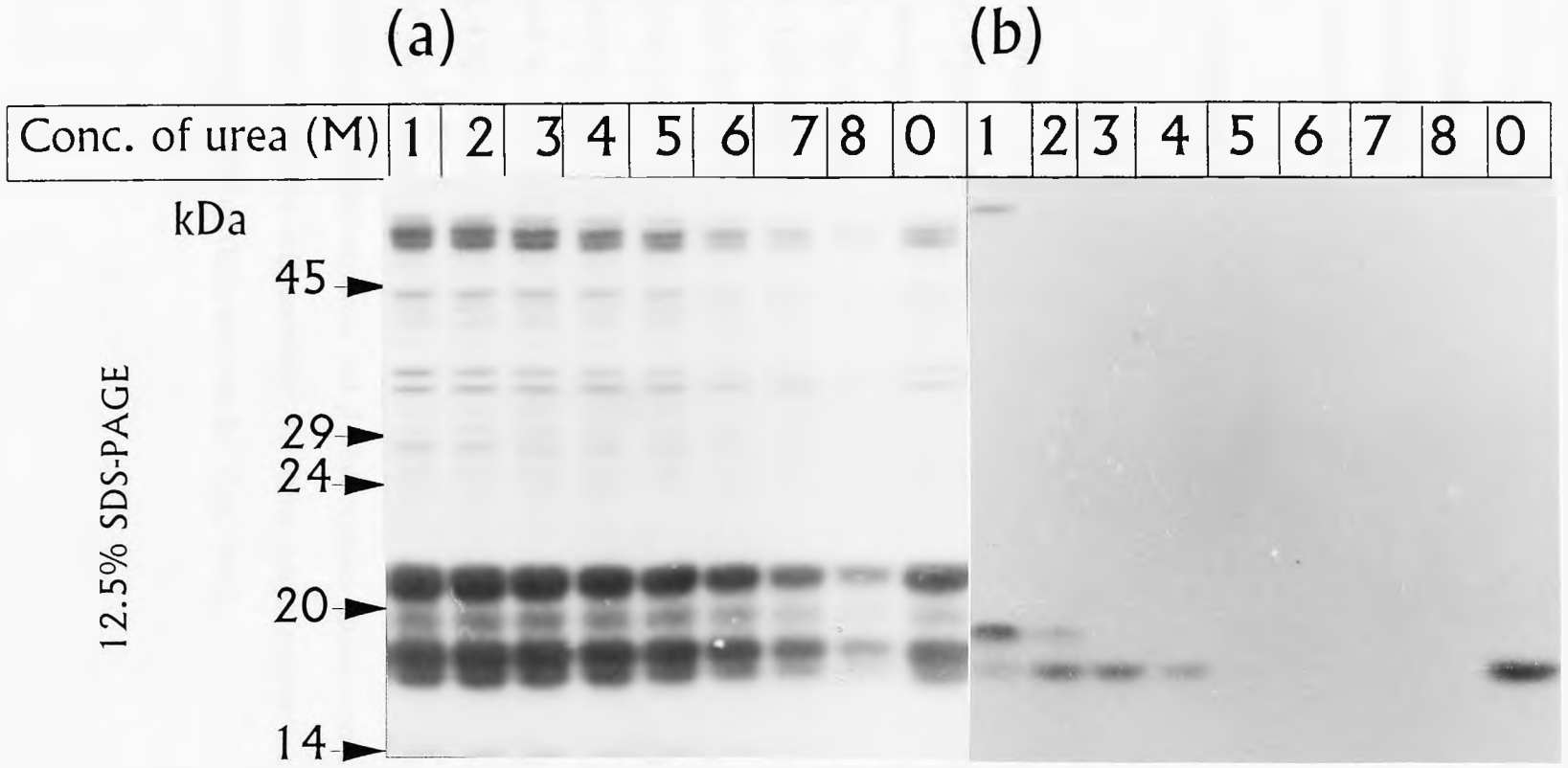

Fig. 4.8. *In vitro* Phosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient in the presence of different concentrations of urea.

The phycobiliprotein-containing fractions of a sucrose density gradient were mixed with different concentrations of urea; proteins were subjected to *in vitro* phosphorylation and were then separated by a 12.5% SDS-PAGE gel and subjected to autoradiography. The different concentrations of urea (final concentration) are as indicated. (a) Coomassie blue stained protein gel, (b) Autoradiograph.

the 20 kDa was not phosphorylated (Fig. 4.8). The phycobiliprotein-containing fractions of the sucrose density gradient were also incubated with different concentration of guanidinium hydrochloride (1 M to 6 M); proteins were then subjected to in vitro phosphorylation. These assays showed that β-phycocyanin could be phosphorylated in the concentrations of 1 M to 4 M (data not shown).

4.4.2. Renaturation of the β-phycocyanin complex

Having established that β-phycocyanin could not be phosphorylated in a concentration of 6 M urea, we tried to find if β-phycocyanin could be phosphorylated again when the urea concentration was diluted. The phycobiliprotein-containing fractions of the sucrose gradient were collected, kept in 8 M urea and incubated at 20°C for 20 min. The urea concentration was then diluted by the addition of different amounts of 20 mM TES buffer, pH 7.5 and incubated at 20°C for another 20 min. Proteins in these samples were subjected to *in vitro* phosphorylation, followed by a pulse-chase reaction by addition of excess unlabelled ATP; proteins were then separated by SDS-PAGE gels and subjected to autoradiography. The result showed that when the urea concentration was diluted to 5 M or less, β-phycocyanin could be phosphorylated and dephosphorylated (Fig. 4.9). This suggested that β-phycocyanin, the β-phycocyanin kinase and phosphatase could be renatured when the urea was diluted. It is currently unknown whether the renaturation of β-phycocyanin and β-phycocyanin kinase was a process of strict self-assembly or assisted self-assembly; the latter might require a molecular chaperone to prevent the misfolding (Ellis and van der Vies, 1991).

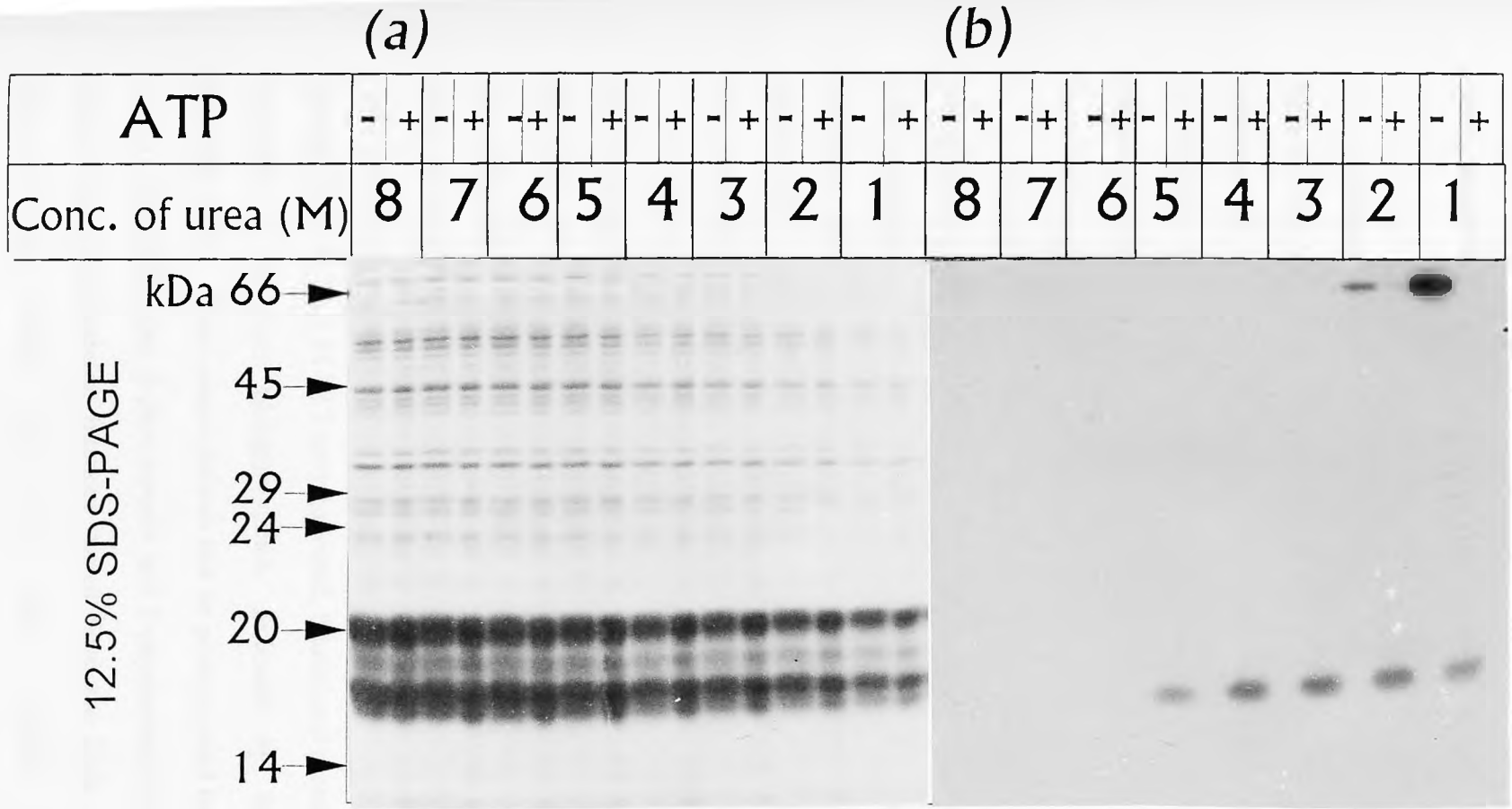

Fig. 4.9. Phosphorylation and dephosphorylation of proteinsin the phycobiliprotein-containing fractions of a sucrose density gradient in the presence of different concentrations of urea.

The phycobiliprotein-containing fractions of a sucrose gradient were kept in 8 M urea (final concentration) for 30 min. The 8 M urea was then diluted and proteins were subjected to *in vitro* phosphorylation, followed by pulse-chase with the addition of excess unlabelled ATP. Proteins were then separated by a 12.5% SDS-PAGE gel and subjected to autoradiography. Different concentrations of urea are as indicated. "+" and "-" refer to with or without cold ATP (1 mM) (a) Coomassie blue stained 12.5% SDS-PAGE gel. (b) Autoradiograph.

4.4.3. Anion exchange chromatography in the presence of urea

Previous experiments had shown that β-phycocyanin was not phosphorylated in 6 M urea, but could be phosphorylated and dephosphorylated again when the urea concentration was diluted, suggesting that β-phycocyanin, β-phycocyanin kinase and the phosphatase could be denatured and renatured. Therefore, we tried to disrupt the β-phycocyanin complex by denaturation of the subunits, which were then separated using anion exchange chromatography in the presence of urea.

The sucrose gradient- and the Mono Q column-purified β-phycocyanin complex was desalted using a Pharmacia PD-10 column and was then incubated in 8 M urea at 20° C for 20 min and loaded on to a Bio-Rad Econo-Pac Q cartridge column which had been previously equilibrated with 20 mM Tris.HCl buffer pH 7.5 containing 8 M urea. A stepwise KCl gradient was used to elute different proteins from the Q cartridge column. The elution pattern showed that a broad protein peak was eluted from the High Q column at 0.1 to 0.3 M KCl and another protein peak was eluted at 0.4 to 0.7 M KCl (Fig. 4.10). Fractions eluted at 0.1 to 0.2 M KCl were pale blue and the SDS-PAGE analysis together with the UV light illumination showed that these fractions mainly consisted of phycobiliproteins (data not shown). Fractions eluted at 0.2 to 0.3 M KCl and fractions eluted at 0.4 to 0.7 M KCl were colourless and did not contain any phycobiliproteins (data not shown). The phycobiliprotein-containing fractions eluted from the Q cartridge column at 0.1 and 0.2 M KCl were collected, diluted and proteins in these fractions were subjected to *in vitro* phosphorylation, followed by SDS-PAGE analysis and autoradiography. These assays showed that no protein could be phosphorylated (data not shown), suggesting that β-phycocyanin and β-phycocyanin kinase were separated. The phycobiliprotein-containing fractions eluted from the High Q column and the other fractions also eluted from the same column were mixed together

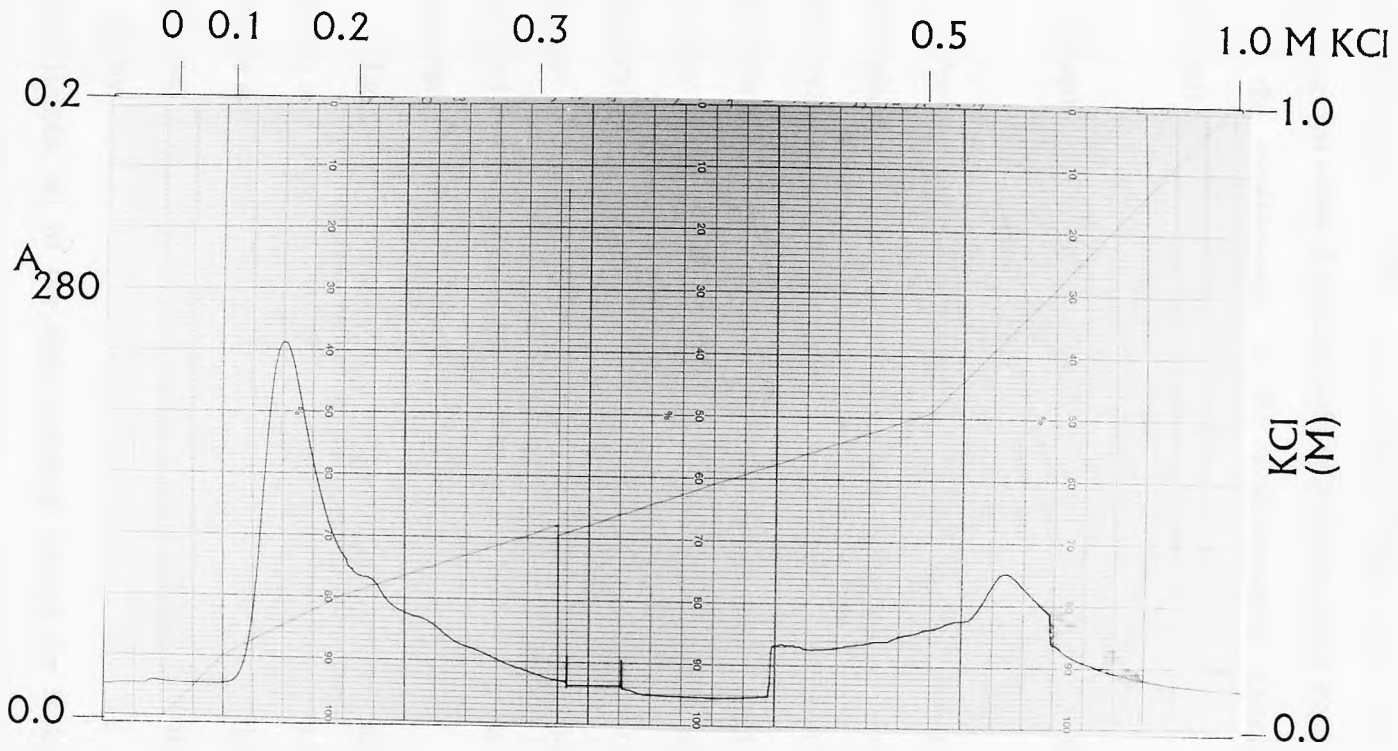

Fig. 4.10. Separation of the urea-denatured β-phycocyanin-containing fractions eluted from the Mono Q column.

The β-phycocyanin-containing fractions eluted from a Mono Q column were desalted, kept in 8 M urea for 30 min and loaded onto a Bio-Rad Econo-Pac High Q column, which had been previous equilibrated with 20 mM Tris-HCl buffer, pH 7.5 containing 8 M urea. A step KCl gradient was used to elute the proteins. Flow rate: 1 ml/min. Concentrations of KCl are as indicated.

and urea was diluted; proteins in these mixed fractions were subjected to *in vitro* phosphorylation by incubation with [γ-^{32}P]ATP, separated by SDS-PAGE and subjected to autoradiography. Still, no protein in these mixed fractions was phosphorylated, suggesting that either β-phycocyanin or β-phycocyanin kinase could not be reconstituted or that the stoichiometry in the β-phycocyanin complex was not correct after reconstitution.

4.5. Phosphorylation of β-phycocyanin in isolated phycobilisomes

Previous experiments had shown that β-phycocyanin, β-phycocyanin kinase and the phosphatase were apparently complexed, consequently we tried to examine whether β-phycocyanin kinase and the phosphatase were located in the phycobilisomes. Intact phycobilisomes were isolated and proteins in the isolated phycobilisomes were subjected to *in vitro* phosphorylation. However, no protein in the isolated phycobilisomes was phosphorylated (data not shown). That β-phycocyanin in the isolated phycobilisomes could not be phosphorylated might result from either β-phycocyanin kinase being inactivated during the process of isolation of phycobilisomes or that there was no kinase in the isolated phycobilisomes.

High concentrations of phosphate buffer (0.75 M NaK_2PO_4, pH 7.5), Triton X-100 (1%) and extended (more than 20-24 hours) exposure to moderate temperatures (20-25°C) are all essential for the isolation of phycobilisomes (Glazer, 1987a) and all of these factors may affect the β-phycocyanin kinase activity. In order to investigate whether or not β-phycocyanin kinase was inactivated during the process of isolation of phycobilisomes, all of the three essential factors for isolating phycobilisomes were examined. (1) The isolated phycobilisomes (10 ml) were dialysed against 20 mM TES buffer, pH 7.5 (5 l) overnight with three changes of TES buffer to dilute the concentration of phosphate buffer; proteins were subjected to *in vitro* phosphorylation by incubation

with [γ-^{32}P]ATP, separation by SDS-PAGE gels and subsequent autoradiography. No protein in the isolated phycobilisomes could be phosphorylated even when the concentration of phosphate buffer was diluted (data not shown), suggesting that either there was no kinase activity in the isolated phycobilisomes or that β-phycocyanin kinase was inactivated by some factor other than the high concentration of phosphate buffer (data not shown). (2) The total membranes, pelleted by ultracentrifugation of the cell-free extracts, were mixed with 1% Triton X-100 (final concentration) and incubated at 20°C for 30 min with gently constant shaking and subjected to a sucrose density gradient ultracentrifugation. Each fraction (1 ml) of the sucrose gradient was collected; proteins in each fraction were phosphorylated *in vitro* and were then subjected to SDS-PAGE analysis and autoradiography. These assays showed that β-phycocyanin was phosphorylated (Fig. 4.11), indicating that Triton X-100 did not affect the activity of β-phycocyanin kinase. (3) The β-phycocyanin-containing fractions eluted from a Mono Q column were incubated at 25°C for up to 4 days; proteins in these fractions were phosphorylated *in vitro*, separated by SDS-PAGE gel and subjected to autoradiography. Assay of β-phycocyanin kinase activity showed that β-phycocyanin was still phosphorylated in the first 3 days (Fig. 4.12), indicating that long time (3 days) exposure to high temperature (25°C) did not inactivate β-phycocyanin kinase. According to the above experiments, the idea that β-phycocyanin kinase was inactivated during the process of isolation of phycobilisomes was not supported; instead, the above experiments strongly suggested that there was no kinase activity in the isolated phycobilisomes.

Fig. 4.12 showed that β-phycocyanin kinase was a stable enzyme, leading us to investigate the stability of the β-phycocyanin kinase in different preparation of the cell-free extracts of *Synechocystis* sp. PCC 6803, which is listed in Table 4.1. When the cells were harvested and frozen at -20°C for at least 9 months or stored at 4°C for at least 3 days, β-phycocyanin kinase was still found to be active when the cells were broken and the kinase activity was tested by phosphorylation of β-phycocyanin *in vitro*. When the

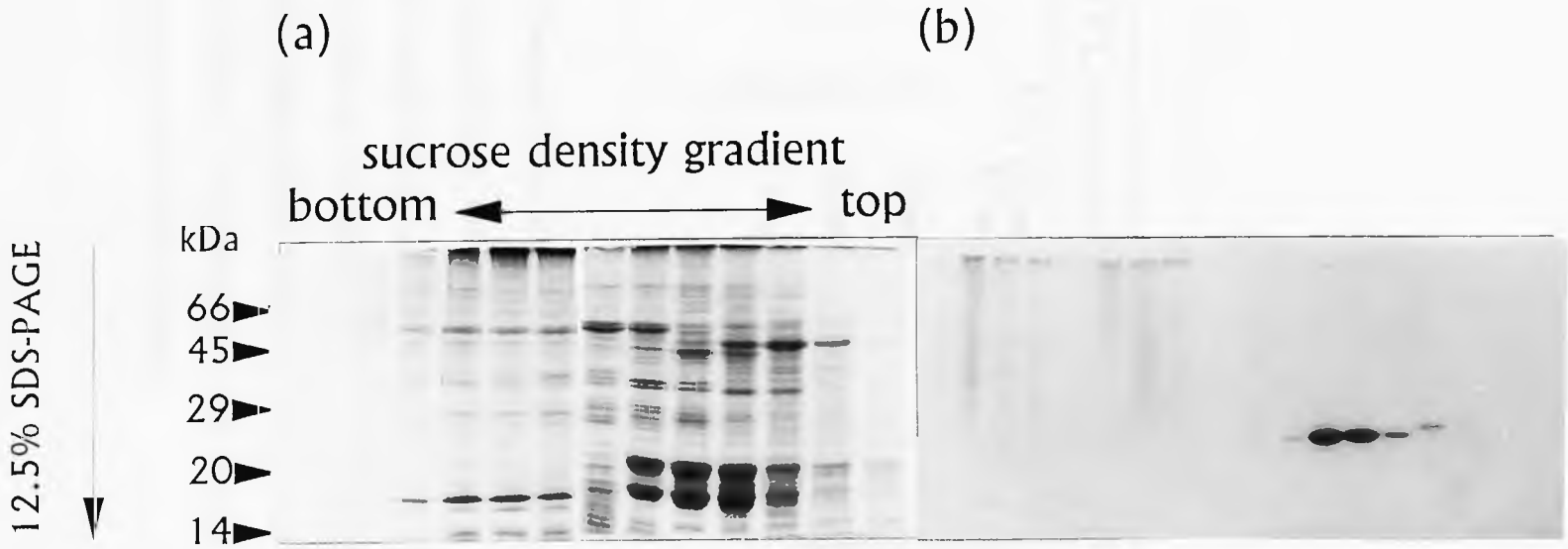

Fig. 4.11. Effects of Triton X-100 on phosphorylation of proteins in the total membranes.

Total membranes of the cell-free extracts were mixed with Triton X-100 (1% final concentration) with constant shaking in the dark for 30 min and then subjected to a sucrose density gradient centrifugation at 130,000 x g at 4°C for 18 hr. Each fraction was collected; proteins in each fraction was subjected to *in vitro* phosphorylation. Proteins were then separated by a 12.5% SDS-PAGE gel and subjected to autoradiography. (a) Coommassie blue stained protein gel. (b) Autoradiograph.

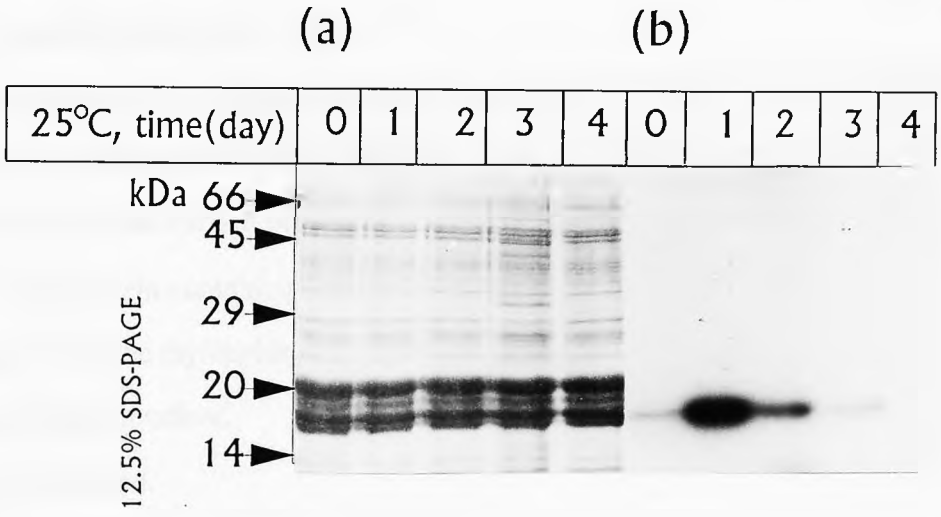

Fig. 4.12. Phosphorylation of proteins in the β-phycocyanin-containing fractions eluted from a Mono Q column and exposed to 25°C for different time intervals.

The β-phycocyanin-containing fractions eluted from the Mono Q column were incubated at 25°C for different time intervals (day) as indicated and proteins were subjected to *in vitro* phosphorylation and were then separated by a 12.5% SDS-PAGE gel and subjected to autoradiography. (a) Coommassie blue stained protein gel. (b) Autoradiograph. Incubation times at 25°C are as indicated.

	whole cells	PB-fractions$^{\Psi}$
at -20°C	at least 9 months	ND*
at 4°C	at least 3 days	at least 1 month
at 25°C	ND*	up to 3 days

Table 4.1 Stability of the β-phycocyanin kinase from fractions of cell-free extracts of *Synechocystis* sp. PCC 6803.

Whole cells and cell-free extracts of *Synechocystis* sp. PCC 6803 were stored at different temperatures for the time intervals indicated. Assay of β-phycocyanin kinase activity in stored whole cells was carried out by *in vitro* phosphorylation of proteins (β-phycocyanin) in the phycobiliprotein-containing fractions of a sucrose density gradient.

$^{\Psi}$ PB-fractions and the thylakoids refer to the phycobiliprotein-containing fractions of a sucrose density gradient.

*ND: not determined.

phycobiliprotein-containing fractions of a sucrose density gradient were collected and stored at 4°C for 1 month or at 25°C for up to 3 days, β-phycocyanin kinase was still found to be active by phosphorylation of β-phycocyanin *in vitro* (Table 4.1).

4.6. Discussion

β-phycocyanin kinase and the phosphatase could not be separated by Mono Q and the Superose 12 columns, and all the experiments showed that both of them co-occurred in a β-phycocyanin complex. However, it is still unknown whether or not the β-phycocyanin kinase and the phosphatase are located in different domains of the same enzyme molecule, like isocitrate dehydrogenase kinase/phosphatase (Nimmo *et al.*, 1984).

When the phycobiliprotein-containing fractions of the sucrose gradient were mixed with 6 M urea *in vitro* kinase assay showed that β-phycocyanin kinase lost its activity; when the urea was diluted, β-phycocyanin kinase activity could be reconstituted. It is still not clear whether or not the reconstitution of the β-phycocyanin kinase activity required molecular chaperones (Ellis and van der Vies, 1991). When the β-phycocyanin-containing fractions eluted from the Mono Q column were desalted and mixed with 8 M urea and were subjected to anion exchange chromatography in the presence of 8 M urea, β-phycocyanin kinase activity could not be reconstituted. The possibility why β-phycocyanin could not be phosphorylated after reconstitution was the stoichiometry of the β-phycocyanin kinase in the β-phycocyanin complex. That is to say, β-phycocyanin could be phosphorylated only when the ratio of β-phycocyanin and the β-phycocyanin kinase was correct. If the stoichiometry was incorrect, it could be that β-phycocyanin and the β-phycocyanin kinase could not form a macromolecular complex and therefore β-phycocyanin could not be phosphorylated. It could also be that the macromolecular complex was formed, but the macromolecular complex was wrong in terms of the structure of the β-phycocyanin complex and hence β-phycocyanin could not be phosphorylated. Another possibility that β-phycocyanin was not phosphorylated after reconstitution was that β-phycocyanin, β-phycocyanin kinase, β-phycocyanin phosphatase and the other components of the complex formed protein aggregates (Babbitt *et al.*, 1990). Our experiments showed that EDTA inhibited the phosphorylation of β-phycocyanin. It

could be that EDTA chelated Mg^{2+} because phosphorylation normally requires Mg^{2+} (or another divalent metal ion such as Mn^{2+}) to form the Mg^{2+}-ATP complex. It could also be that EDTA inhibited the formation of protein aggregates by chelating some divalent cations because formation of protein aggregates normally requires Ca^{2+}. When these proteins occurred in the aggregate, β-phycocyanin kinase expressed the kinase activity; contrarily, if the protein aggregate did not occur, β-phycocyanin kinase lost its activity. However, which hypothesis is correct has still to be proven. There were at least two proteins having nearly the same size and nearly the same isoelectric point as β-phycocyanin (Fig. 4.1). Whether or not these proteins occurred in the β-phycocyanin complex or the protein aggregate and the identities of these proteins are still to be determined.

When the β-phycocyanin-containing fractions eluted from the Mono Q column were incubated at 25°C for 3 days, β-phycocyanin kinase was still active (Fig. 4.12), indicating that this kinase was a stable enzyme. The high stability of β-phycocyanin kinase might be due to its co-occurrence with β-phycocyanin in the macromolecular complex because the presence of the substrates has a specific stabilising effect on enzymes (Dixon *et al.*, 1979) and therefore β-phycocyanin was still active after incubation at 25°C for up to 3 days. It was also noticeable that the intensity of the labelling of β-phycocyanin increased after incubation at 25°C for 24 hr. The increase of the intensity of the labelling of β-phycocyanin might be due to the exposure of the catalytic site of the β-phycocyanin kinase to the environment and to the substrate during the process of denaturation of the protein molecule (Shoichet *et al.*, 1995); therefore, after incubation at 25°C for 24 hr, β-phycocyanin was strongly phosphorylated and then β-phycocyanin kinase gradually lost its activity because of denaturation.

Chapter 5

Physiological Functions of the Phosphorylation of β-phycocyanin and Molecular Biology of β-phycocyanin

5.1. Introduction

Photosynthetic proteins in pea chloroplasts have been found to be phosphorylated in the light (Bennett, 1977) and dephosphorylated in the dark (Bennett, 1980). That phosphorylation of photosynthetic proteins results in redistribution of excitation energy has been widely accepted (for review see Bennett, 1991; Allen, 1992). Cyanobacteria have also been shown to exhibit state-transitions with the concomitant phosphorylation of a number of thylakoid proteins (Sanders and Allen, 1987, and 1988; Harrison *et al.*, 1991; Race and Gounaris, 1993).

Previous experiments (Chapter 3) had shown that an 18 kDa was phosphorylated and the kinase activity of cell-free extracts was also demonstrated. The phosphorylated 18 kDa protein was tentatively identified as β-phycocyanin in which a serine residue(s) was modified. However, which particular serine was phosphorylated is still not determined. Phosphorylation and dephosphorylation of β-phycocyanin were insensitive to standard inhibitors of protein kinases and phosphatase.

Consequently, attempts to confirm that the 18 kDa protein was β-phycocyanin and to locate the phosphorylation site were made. In addition, we also tried to establish the physiological role(s) of the phosphorylation of β-phycocyanin, especially focusing on the relationship between phosphorylation of β-phycocyanin and photosynthesis.

To investigate the physiological function(s) of the phosphorylation of β-phycocyanin, two approaches, in terms of the biochemistry and the molecular biology, would be used for achieve this goal. For the biochemical approach, the effects of light on phosphorylation of β-phycocyanin and the absorption and fluorescence characteristics of β-phycocyanin would be investigated. In terms of molecular biology, site-directed mutations, preventing β-phycocyanin phosphorylation, would be created, thereby providing the information of the physiological function(s) of the phosphorylation of β-phycocyanin. To confirm that the 18 kDa protein was β-phycocyanin, the β-phycocyanin

gene (*cpcB* gene) would be cloned and sequenced and the predicted amino acid sequence deduced from the *Synechocystis* sp. PCC 6803 *cpcB* gene and the N-terminal amino acid sequence of the 18 kDa protein would be compared. Creation of the site-directed mutations would provide not only the information of the physiological role of the phosphorylation of β-phycocyanin, but also about the phosphorylation site of β-phycocyanin

5.2. Effects of spectral quality of the light on phosphorylation and dephosphorylation of β-phycocyanin

Different qualities of light can affect phosphorylation of photosynthetic proteins both *in vivo* and *in vitro* (for review see Bennett, 1991; Allen, 1992). Consequently, the effects of light on phosphorylation of β-phycocyanin were examined.

5.2.1 Effects of blue light and orange light on phosphorylation β-phycocyanin

Blue light with a wavelength greater than 700 nm is predominantly absorbed by PS I. Orange light with wavelength between 550 nm to 620 nm, is predominantly absorbed by PS II. Besides, blue light has been reported to activate protein kinase activities in higher plants (Reymond *et al.*, 1992a); therefore, the effects of light with different spectral qualities on phosphorylation were investigated.

The phycobiliprotein-containing fractions of the sucrose gradient were incubated at 25°C and illumination with blue light or orange light for 60 min. Proteins were then subjected to phosphorylated *in vitro* which was then followed by a pulse-chase reaction by the addition of excess unlabelled ATP and further incubation in orange light or blue light for 10 min. Proteins were then subjected to SDS-PAGE analysis and autoradiography. These assays showed that blue light and orange light did not affect

phosphorylation and dephosphorylation of β-phycocyanin, indicating that phosphorylation and dephosphorylation of β-phycocyanin were light-independent *in vitro* (Fig. 5.1). Phosphorylation of the 20 kDa and the 56 kDa polypeptides was also insensitive to the blue light and orange light. Previous experiments (Section 3.8.1) had shown that the 20 kDa polypeptide was not subjected to dephosphorylation.

5.2.2. Effects of light-dark shifts on phosphorylation of β-phycocyanin

Transition between light and dark had also been reported to have some influence on phosphorylation and dephosphorylation of proteins in both eukaryotic and prokaryotic cells (Bennett, 1977; Bennett, 1980; Harrison *et al.*, 1991; Warner and Bullerjahn, 1994). Preincubation of the thylakoid membranes of *Synechocystis* sp. PCC 6803 in PS I or PS II light, followed by incubation in the white light or the dark also led to phosphorylation of several membrane proteins *in vitro* (Silman and Mann, unpublished data). Consequently, the effects of light-dark shifts on phosphorylation of β-phycocyanin were also examined *in vitro*.

The phycobiliprotein-containing fractions of the sucrose gradient were illuminated under PS I light (710 nm) or PS II (580 nm) light for 60 min at 25°C; proteins were then subjected to phosphorylation *in vitro* in the white light or dark for 10 min, separated by SDS-PAGE and subjected to autoradiography. As shown in Fig. 5.2, the light-dark shifts did not affect phosphorylation and dephosphorylation of β-phycocyanin.

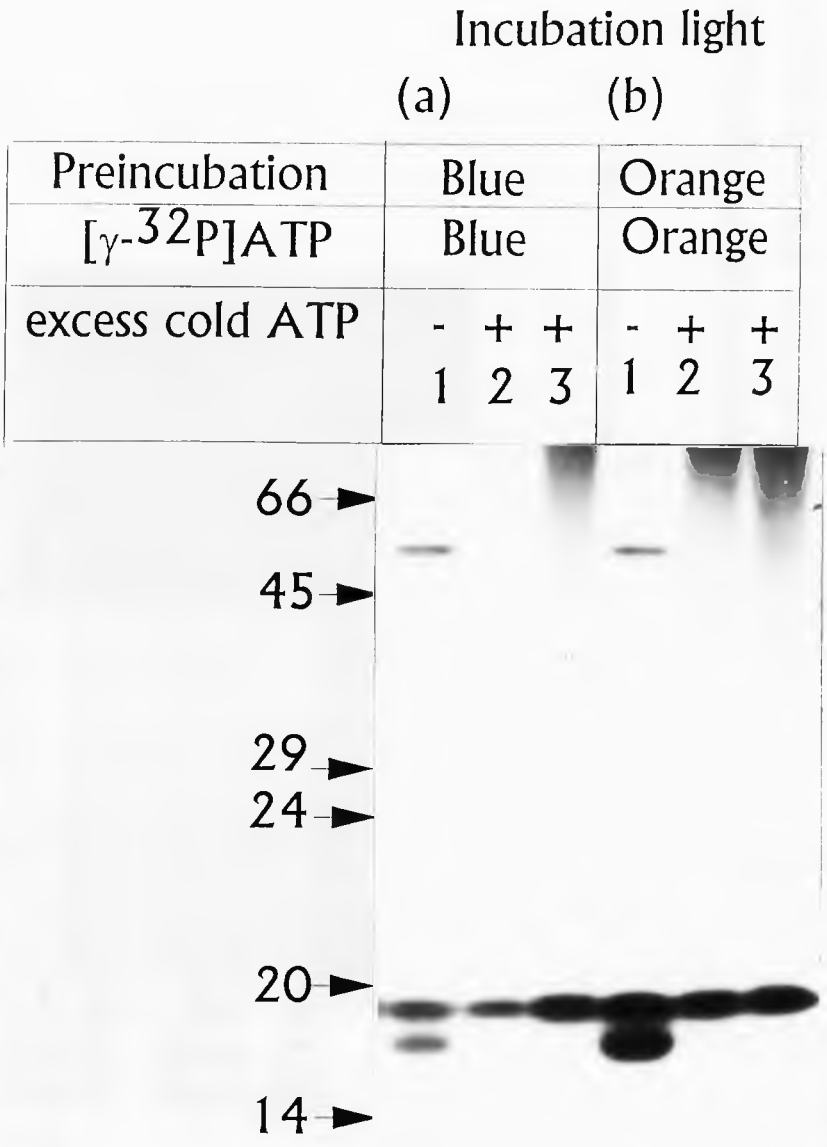

Fig. 5.1. Effects of orange light and blue light on phosphorylation and dephosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose gradient.

The phycobiliprotein-containing fractions of the sucrose gradient were illuminated in the blue light or the orange light for 1 hr. Proteins were subjected to *in vitro* phosphorylation, followed by pulse-chase by the addition of excess unlabelled ATP and illuminated in the blue light or the orange light simultaneously. Proteins were separated by 12.5% SDS-PAGE gel and subjected to autoradiography. The autoradiograph shows *in vitro* phosphorylation in the blue light (a) or the orange light (b). (1) without pulse-chase; (2) with pulse-chase in the blue light; (3) with pulse-chase in the orange light.

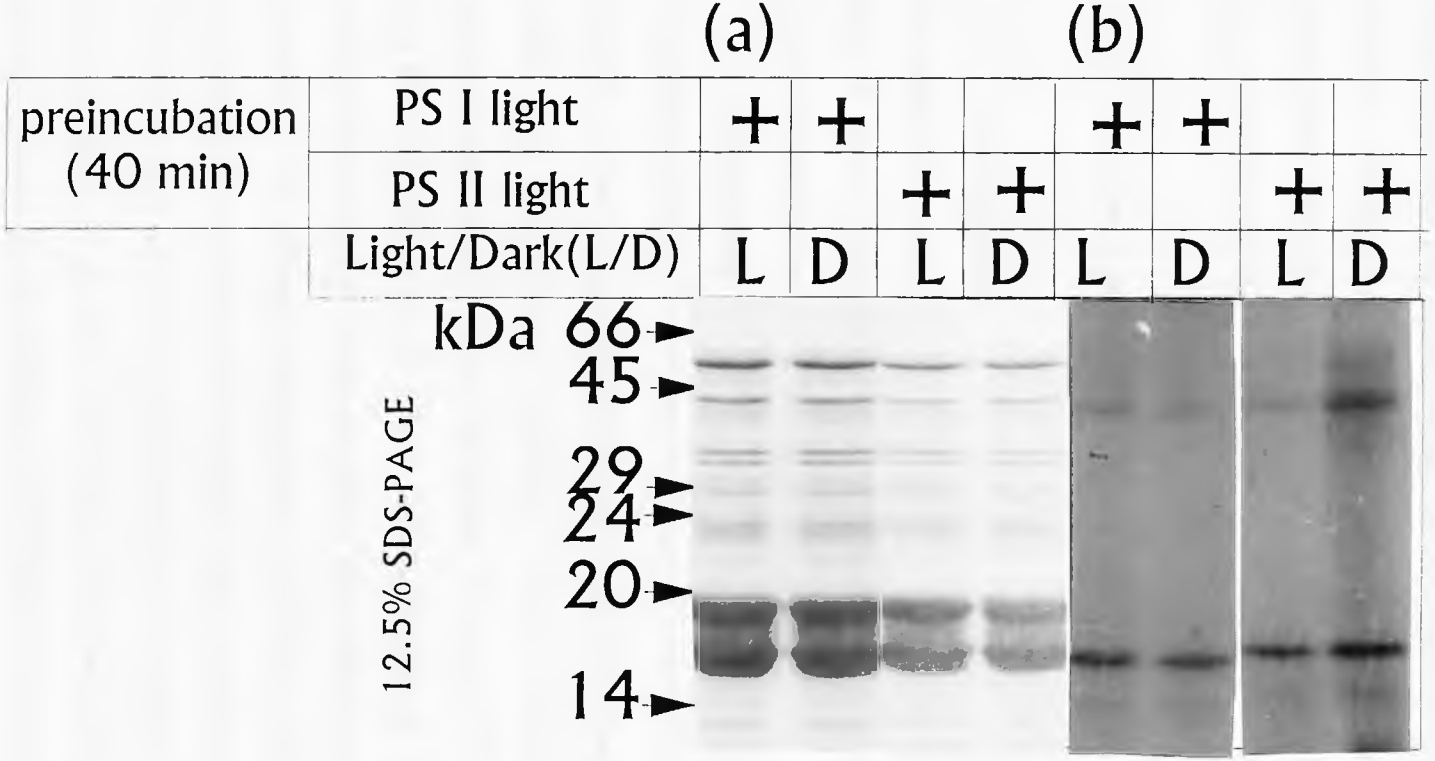

Fig. 5.2. Effects of light-dark shifts and different qualities of light on phosphorylation and dephosphorylation of proteins in the phycobiliprotein-containing fractions of a sucrose density gradient.

The phycobiliprotein-containing fractions of a sucrose density gradient were illuminated in the PS I light or PS II light for 1 hr; proteins were then subjected to *in vitro* phosphorylation in white light or in the dark. Proteins were separated by 12.5% SDS-PAGE gel and subjected to autoradiography. (a) Coomassie blue stained protein gel; (b) Autoradiography. Abbreviations: L, light; D, dark. "+" refers to illumination of light.

5.3. Spectroscopy of β-phycocyanin

Phycobiliproteins can absorb certain spectral quality of light and strongly fluoresce, therefore, effects of phosphorylation on the spectrophotometric properties of β-phycocyanin were investigated by measuring the absorption spectra and the fluorescence spectra of β-phycocyanin.

5.3.1. Absorption spectra of β-phycocyanin

Phycobiliproteins can absorb certain wavelengths of light (for review see Grossman 1993a, and 1993b). In order to investigate if β-phycocyanin could still absorb light, in terms of spectral quality and quantity, after phosphorylation, the absorption spectra of β-phycocyanin samples were measured.

β-phycocyanin-containing fractions eluted from a Mono Q column were subjected to spectrophotometry. The absorption spectra of proteins in the β-phycocyanin-containing fractions showed a broad absorption with a maximum ranging between 615 to 620 nm (Table 5.1), which was in accordance with the published maximum absorption of phycocyanobilin (for review see Grossman 1993a, and 1993b). The concentration of ATP in a cell is of the order of 2 to 4 mM so proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column were subjected to *in vitro* phosphorylation by incubation with different concentrations of ATP (0.5 μM to 4 mM), followed by the measurement of their absorption spectra. These absorption spectra also showed a broad absorption with a maximum ranging between 615 to 620 nm (Table 5.1). Besides, *in vitro* phosphorylation of proteins in the β-phycocyanin-containing fraction eluted from the Mono Q column by the addition of [γ-^{32}P]ATP showed that only β-phycocyanin was phosphorylated (data not shown) confirming that the phosphorylation of β-phycocyanin did not significantly alter the absorption spectrum.

ATP conc.	absorption λ (nm)/ maximum absorption (peak height)	
	before phosphorylation	10 min after phosphorylation
0.5 μM	615.2/0.023	615.2/0.028
1.0 μM	620.9/0.031	615.2/0.030
5.0 μM	615.2/0.031	616.0/0.024
10 μM	619.2/0.028	614.7/0.026
50 μM	615.2/0.029	620.8/0.027
100 μM	620.0/0.023	618.4/0.024
500 μM	617.6/0.026	620.0/0.022
1 mM	615.2/0.020	617.6/0.026
2 mM	615.2/0.021	619.2/0.026
3 mM	613.6/0.020	615.2/0.020
4 mM	615.2/0.023	615.2/0.028

Table 5.1. Absorption maxima of proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column.

The phycobiliprotein-containing fractions of a sucrose density gradient were pooled and loaded on a Mono Q column. The β-phycocyanin-containing fractions eluted from the Mono Q column were subjected to *in vitro* phosphorylation by addition of different concentrations of ATP (as indicated). The absorption spectra of the proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column were measured before and after phosphorylation. The wavelengths of the maximum absorption and the peak height are as indicated.

5.3.2. Fluorescence emission spectra of β-phycocyanin

Phosphorylation of photosynthetic proteins results in changes of chlorophyll fluorescence (Bennett et al., 1980; Horton and Black, 1982; McCormac *et al.*, 1994), which has been correlated with redistribution of excitation energy (for review see Bennett, 1991; Allen, 1992). Therefore, the fluorescence emission spectra of β-phycocyanin were examined, thereby investigating if phosphorylation could affect the fluorescence of β-phycocyanin and excitation energy transfer.

The phycobiliprotein-containing fractions of a sucrose density gradient were pooled and loaded on to a Mono Q column and proteins were eluted from the Mono Q column. Proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column were or were not subjected to *in vitro* phosphorylation, followed by measurement of the fluorescence emission spectrum with excitation at 600 nm. Before *in vitro* phosphorylation, proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column showed a fluorescence emission spectra ranging between 620 nm to 720 nm with a maximum at 650 nm (Fig. 5.3c). The maximum fluorescence emission decreased 25% and 50% at 5 and 10 min after *in vitro* phosphorylation, respectively (Fig. 5.3b and 5.3a). SDS-PAGE analysis and autoradiography of the *in vitro* phosphorylation of these β-phycocyanin-containing fractions eluted from the Mono Q column showed that only β-phycocyanin was phosphorylated, confirming that the decrease of fluorescence emission resulted from the phosphorylation of β-phycocyanin (data not shown). When EDTA was added to these β-phycocyanin-containing fractions eluted from the Mono Q column to inhibit phosphorylation of β-phycocyanin, as expected, the fluorescence emission spectra were not altered (Fig. 5.4). Therefore, phosphorylation of β-phycocyanin resulted in fluorescence quenching, suggesting that the excitation energy was lost via non-radiative dissipation.

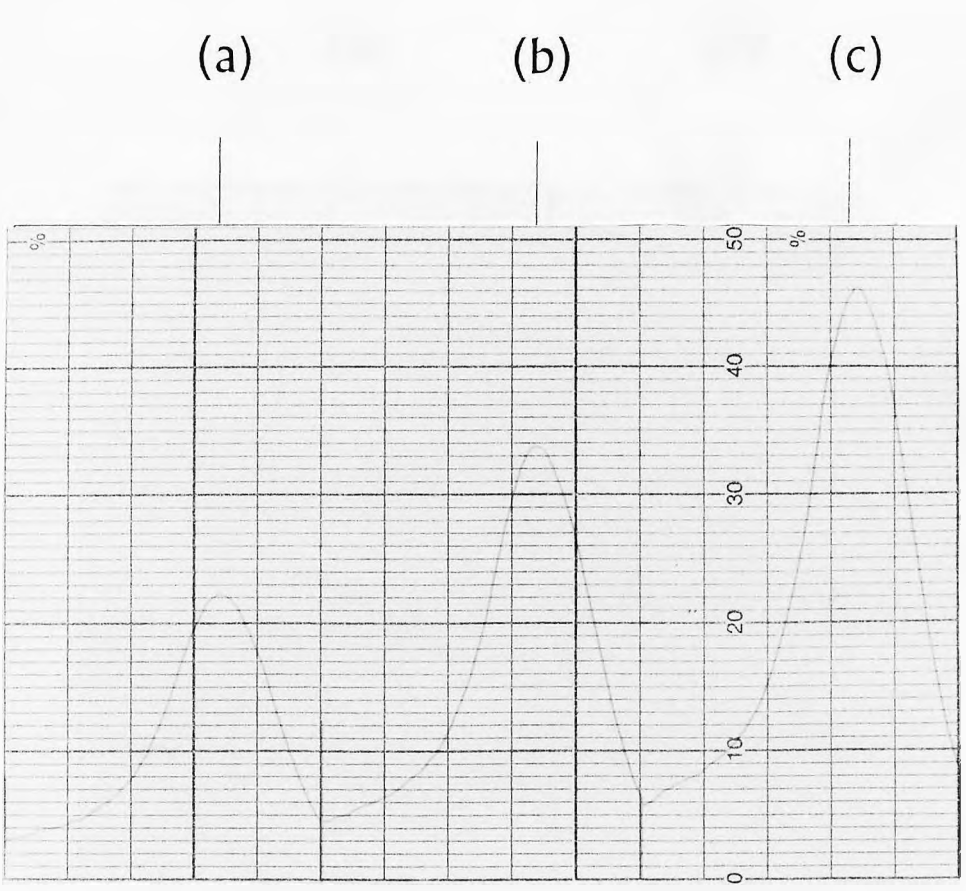

Fig. 5.3. Effect of phosphorylation on fluorescence of phosphorylation of proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column.

The phycobiliprotein-containing fractions of the sucrose density gradient were pooled and loaded on an FPLC Mono Q column. Proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column were subjected *in vitro* phosphorylation for different time intervals. Fluorescence emission spectra of protein before and after phosphorylation were recorded using Perkin-Elmer LS-5 fluorescence spectrometer. Excitation: 600 nm. Fluorescence spectral range: 620-720 nm. (a) 10 min after phosphorylation; (b) 5 min after phosphorylation; (c) before phosphorylation.

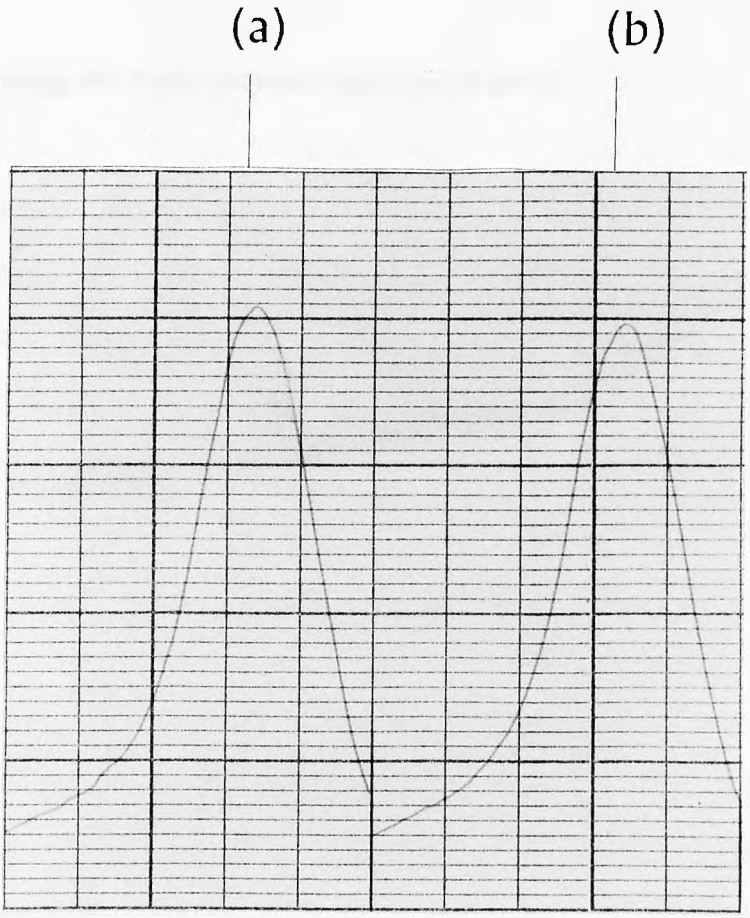

Fig. 5.4. Effect of EDTA on fluorescence of proteins in the β-phycocyanin-containing fractions eluted from a Mono Q column.

The phycobiliprotein-containing fractions of the sucrose density gradient were pooled and loaded on an FPLC Mono Q column. Proteins in the β-phycocyanin-containing fractions eluted from the Mono Q column were incubated with or without EDTA. Proteins were then subjected to *in vitro* phosphorylation for 10 min. Fluorescence emission spectra of proteins were recorded using a Perkin-Elmer LS-5 fluorescence spectrometer. Excitation: 600 nm. Fluorescence spectral range: 620-720 nm. (a) incubation with EDTA. (b) incubation without EDTA.

5.4. Attempts at cloning the β-phycocyanin gene (*cpcB* gene)

Creation of site-directed mutations, preventing β-phycocyanin phosphorylation, could provide the information about the physiological functions phosphorylation of β-phycocyanin. Several β-phycocyanin gene (*cpcB* gene) sequences have been reported for cyanobacteria and eukaryotic red algae (Troxler *et al.*, 1995; Wilson *et al.*, 1991; Pilot and Fox, 1984; Belknap and Haselkorn, 1987); however, the *Synechocystis* sp. PCC 6803 *cpcB* gene sequence had not yet been published. Therefore, cloning and sequencing of the *Synechocystis* sp. PCC 6803 *cpcB* gene would be necessary.

Three methods could potentially be used to clone the *Synechocystis* sp. PCC 6803 *cpcB* gene. Firstly, restriction digestion of the *Synechocystis* sp. PCC genome, and probing with the *Synechococcus* sp. PCC 7002 *cpcB* gene. Secondly, amplification of the *Synechocystis* sp. PCC 6803 *cpcB* gene by the polymerase chain reaction (PCR). Thirdly, screening of a cosmid library containing the *Synechocystis* sp. PCC 6803 genome with the *Synechococcus* sp. PCC 7002 *cpcB* gene.

5.4.1. Restriction digestion of *Synechocystis* sp. PCC 6803 genomic DNA

Synechocystis sp. PCC 6803 genomic DNA was prepared and digested with different kinds of restriction enzymes (*BamH1*, *EcoR1*, *HindIII*, *Kpn1*, *Pst1*, *Sal1*, *Sst1* and *Xba1*) to find out which restriction enzyme(s) would give a better digestion result. The restriction digestion showed that the *Synechocystis* sp. PCC 6803 genomic DNA was well digested by *HindIII*, *Pst1* and *Xba1* (data not shown). DNA fragments of the restriction digestion of the *Synechocystis* sp. PCC 6803 genomic DNA were blotted onto a Hybond-N filter which was then probed with the *Synechococcus* sp. PCC 7002 *cpcB* gene radiolabelled with [α-^{32}P]ATP by random priming and subjected to autoradiography. Autoradiography of the Hybond-N filter showed in contrast to the

positive control, a very weak hybridisation signal with the *Synechocystis* sp. PCC 6803 genomic DNA (data not shown), suggesting that the *Synechocystis* sp. PCC 6803 *cpcB* gene was not very homologous to the *Synechococcus* sp. PCC 7002 *cpcB* gene.

5.4.2. Amplification of the *Synechocystis* sp. PCC 6803 *cpcB* gene by PCR

Since the *Synechocystis* sp. PCC 6803 *cpcB* gene was not very homologous to the *Synechococcus* sp. PCC 7002 *cpcB* gene, the *Synechocystis* sp. PCC 6803 *cpcB* gene was amplified by means of PCR for sequencing. Two conserved regions of β-phycocyanin from several cyanobacterial strains (from Val-9 to Asp-14 and from Phe-165 to Ala-170) were chosen to design the PCR primers. The sequence of the forward primer was 5'-ACG TGG ATC CGT ATC TCA AGC TGA TGC-3' and the sequence of the reverse primer was 5'-ACG TGG TAC CTA AGT CTC CCG TAG ACC-3'. The best conditions for the PCR were empirically established and each cycle was composed of 92°C for 60 sec, 40°C for 60 sec and then 72°C for 60 sec, and the PCR was finished after 30 cycles. When PCR was finished the PCR products were separated by agarose gel electrophoresis and the DNA fragments were visualised by UV light, and a PCR product of the expected size of 500 bp was obtasined (Fig. 5.5).

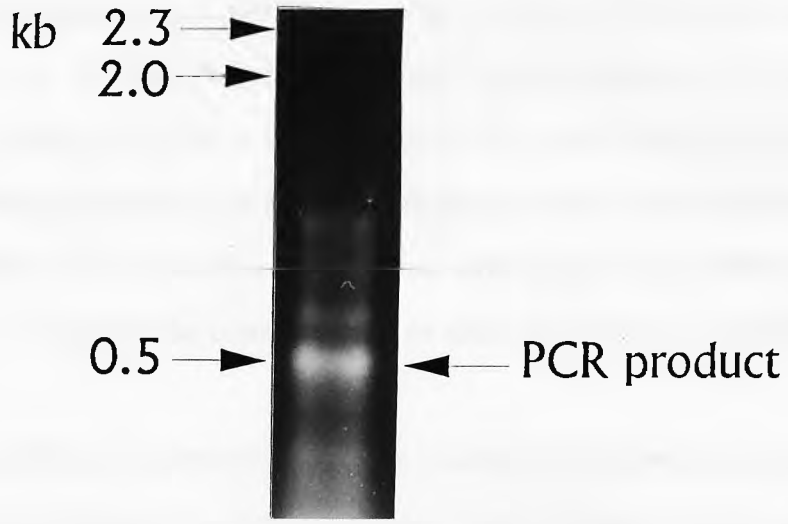

Fig. 5.5. PCR amplification of *Synechocystis* sp. PCC 6803 *cpcB* gene.

Synechocystis sp. PCC 6803 chromosomal DNA was isolated and used as a template of polymerase chain reaction (PCR). The sequence of the forward primer was 5'-ACG TGG ATC CGT ATC TCA AGC TGA TGC-3' and the sequence of the reverse primer was 5'-ACG TGG TAC CTA AGT CTC CCG TAG ACC-3'. PCR was carried out for 30 cycles, each cycle consisting of 92°C for 1 min, 40°C for 1 min and 72°C for 1 min in the presence of *Taq* polymerase (5 units). The PCR product was separated using a 0.7% agarose gel. The arrow indicates the major PCR product.

5.4.3. Screening of cosmid library containing *Synechocystis* sp. PCC 6803 genome

Recently the physical and genetic map of the *Synechocystis* sp. PCC 6803 genome have been reported (Churin *et al.*, 1995) and a cosmid library of 760 clones containing the *Synechocystis* sp. PCC 6803 genome has also been constructed (S. Shestakov, personal communication). The vector for constructing the cosmid library was Lobrist 6. The capacity of the gene library was about 9 equivalents of the *Synechocystis* sp. PCC 6803 genome. Each clone contained an insertion averaging 39 kb. While the PCR experiments were in progress the cosmid library (a kind gift of Prof. S. Shestakov) was sent to us.

The cosmid library was hybridised with the radiolabelled *Synechococcus* sp. PCC 7002 *cpcB* gene and subjected to autoradiography. Autoradiography of the *cpcB* gene probed cosmid library showed that 9 clones (A1#1, A1#2, A5#5, B1#1, B2#3, B11#4, E9#2, F5#5 and H9#6) were hybridised with the *Synechococcus* sp. PCC 7002 *cpcB* gene (data not shown); however, the background of the autoradiograph was too high to recognise which one(s) of the nine clones was the right one(s) containing the *Synechocystis* sp. PCC 6803 *cpcB* gene. In order to further confirm which cosmid clone(s) contained the *Synechocystis* sp. PCC 6803 *cpcB* gene, the 9 cosmid clones were cultured in LB medium containing kanamycin (25μg/ml). DNA from each clone was isolated by the miniprep method and digested with different restriction enzymes (*SalI* and *HindIII*); the digested DNA fragments were separated by agarose gel electrophoresis, blotted onto a Hybond-N filter, which was then probed with the *Synechococcus* sp. PCC 7002 *cpcB* gene and subjected to autoradiography. Autoradiography of the Hybond-N filter showed that the E9#2 clone hybridised with the *Synechococcus* sp. PCC 7002 *cpcB* gene, confirming that this E9#2 clone contained the *Synechocystis* sp. PCC 6803 *cpcB* gene (Fig. 5.6). The E9#2 clone DNA was double digested with different kinds of restriction enzymes and the DNA fragments were separated by agarose gel, blotted onto a

Hybond-N filter, which was then probed with 7002 *cpcB* gene and subjected to autoradiography. Autoradiography of the Hybond-N filter showed that a 2.5 kb fragment of the E9#2 DNA digested by *Pst1* and *Xba1* was labelled, indicating that this 2.5 kb *Pst1-Xba1* fragment contained the *Synechocystis* sp. PCC 6803 *cpcB* gene (data not shown).

5.4.4. Comparison of the amino acid sequence deduced from the *Synechocystis* sp. PCC 6803 *cpcB* gene with the N-terminal amino acid of the 18 kDa protein

While our PCR experiments and the cosmid clone screening experiments were still in progress the *Synechocystis* sp. PCC 6803 *cpcB* gene was cloned and sequenced (L. Anderson, personal communication) and the sequence made available to us. Cloning of the *Synechocystis* sp. PCC 6803 *cpcB* gene involved restriction digestion of the *Synechocystis* sp. PCC 6803 genomic DNA with *Xba1,* and a 3.8 kb *Xba1* fragment was cloned into plasmid pBluescriptII to construct the plasmid pPC338, which contained the *cpcB*, *cpcA*, *cpcH* and part of the *cpcI* genes, which were all sequenced (L. Anderson, personal communication).

The sequence of the *Synechocystis* sp. PCC 6803 *cpcB* gene is shown in Fig. 5.7. The predicted amino acid sequence of β-phycocyanin of *Synechocystis* sp. PCC 6803 is shown in Fig. 5.8. The N-terminal amino acid sequence of the 9 kDa fragment of V8 protease digested 18 kDa protein and that of the β-phycocyanin deduced from the *Synechocystis* sp. PCC 6803 *cpcB* gene are identical except that the first amino acid of the predicted *Synechocystis* sp. PCC 6803 β-phycocyanin was methionine rather than valine.

Fig. 5.6. Screening of 9 cosmid clones containing the *Synechocystis* sp. PCC 6803 genomic DNA, probed with the *Synechococcus* sp.PCC 7002 *cpcB* gene, labelled by random priming.

DNAs of the cosmid library clones found to hybridise with *Synechococcus* sp. PCC 7002 *cpcB* gene were isolated using the miniprep method. The isolated DNAs were separated on a 0.7% agarose gel, blotted onto a Hybond-N membrane and probed with the *Synechococcus* sp. 7002 *cpcB* gene. Each track of the autoradiograph of the Southern blot is as follows: 1, positive control (plasmid pAQPR1); 2, A1#1; 3, A1#2; 4, A5#5; 5, B1#1; 6, B2#3; 7, F5#5; 8, E9#2.

```
  1  ATGTTCGACG TATTCACTCG GGTTGTTTCC CAAGCTGATG CTCGCGGCGA
 51  GTACCTCTCT GGTTCTCAGT TAGATGCTTT GAGCGCTACC GTTGCTGAAG
101  GCAACAAACG GATTGATTCT GTTAACCGCA TCACCGGTAA TGCTTCCGCT
151  ATCGTTTCCA ACGCTGCTCG TGCTTTGTTC GTTGAACAGC CCCAATTAAT
201  CCAACCCGGT GGAAACGCCT ACACCAGCCG TCGTATGGCT GCTTGTTTGC
251  GTGACATGGA AATCATCCTC CGCTATGTTA CCTACGCAAC CTTCACCGGC
301  GACGCTTCCG TTCTAGAAGA TCGTTGCTTG AACGGTCTCC GTGAAACCTA
351  CGTTGCCCTG GGTGTTCCCG GTGCTTCCGT AGCTGCTGGC GTTCAAAAAA
401  TGAAAGAAGC TGCCCTGGAC ATCGTTAACG ATCCCAATGG CATCACCCGT
451  GGTGATTGCA GTGCTATCGT TGCTGAAATC GCTGGTTACT TCGACCGCGC
501  CGCTGCTGCC GTAG
```

Fig. 5.7. DNA sequences of the *Synechocystis* sp. PCC 6803 *cpcB* gene.

The *Synechocystis* sp. PCC 6803 chromosomal DNA was restriction digested with *Xba1*. A 3.8 kb *Xba1* fragment was cloned into plasmid pBluescriptII to construct the plasmid pPC338, which contained *cpcB*, *cpcA*, *cpcH* and part of *cpcI* genes and the genes were then sequenced (L. Anderson, personal communication).

```
          1                                                    50
6803pcb   .MFDVFTRVV SQADARGEYL SGSQLDALSA TVAEGNKRID SVNRITGNAS
9 kDa pep .VFDVFTRVV SQADA
          51                                                   100
6803pcb   AIVSNAARAL FVEQPQLIQP GGNAYTSRRM AACLRDMEII LRYVTYATFT

          101                                                  150
6803pcb   GDASVLEDRC LNGLRETYVA LGVPGASVAA GVQKMKEAAL DIVNDPNGIT

          151                        174
6803pcb   RGDCSAIVAE IAGYFDRAAA AVA.
```

Fig. 5.8. Comparison of the N-terminal amino acid sequence of the 9 kDa fragment of the V8 digested 18 kDa protein in the phycobiliprotein-containing fractions of the sucrose gradient with the amino acid sequence of β-phycocyanin predicted from the DNA sequence of the *Synechocystis* sp. PCC 6803 *cpcB* gene (L. Anderson, personal communication).

5.5. Discussion

Phosphorylation of β-phycocyanin in the phycobiliprotein-containing fractions of the sucrose gradient was light-independent *in vitro*. However, Harrison *et al.* (1991) found that phosphorylation of an 18.5 kDa, which was proposed to be one of the components of the phycobilisome (Sanders and Allen, 1987) and was tentatively identified as β-phycocyanin (Harrison, 1990) was light-dependent and this 18.5 kDa protein was dephosphorylated in the dark. Whether phosphorylation of β-phycocyanin was light-dependent or light-independent might be because of the presence of the thylakoid membranes. In our experiments, β-phycocyanin was from the phycobiliprotein-containing fractions of the sucrose density gradient, which were the thylakoid membrane-free fractions. However, in the experiments of Harrison *et al.* (1991), the 18.5 kDa protein was located in the crude total membrane fraction. When the thylakoid membranes were illuminated with light, the plastoquinone pools located in the electron transport chain in the thylakoid membranes would have become reduced and therefore a kinase(s) might have been activated (Allen *et al.*, 1981), resulting in protein phosphorylation. When the thylakoid membranes were incubated in the dark, the plastoquinone pool would have become oxidised and therefore the protein kinase migh have been inactivated, leading to protein dephosphorylation because protein phosphatase activity remained constant and was independent of redox control (Allen *et al.*, 1981; Sanders and Allen, 1988). Thus, the 18.5 kDa protein would be phosphorylated in the light and dephosphorylated in the dark in the thylakoid preparation. It could be that the thylakoid membranes, or more precisely, the plastoquinone pool (Allen et al., 1981; Allen and Horton, 1981) and the cytochrome $b_{6/f}$ complex (Gal *et al.*, 1988) of the electron transport chain in the thylakoid membranes, are responsible for the on/off control of β-phycocyanin kinase. Thus without the thylakoid membranes, the β-phycocyanin kinase would be always activated and this would be consistent with previous experiments (see Section 3.8) which showed that

phosphorylation and dephosphorylation of β-phycocyanin occurred simultaneously because both β-phycocyanin kinase and phosphatase were active. When the phycobiliprotein-containing fractions of the sucrose gradient were incubated with the thylakoid membranes phosphorylation of β-phycocyanin became light-dependent (N. J. Silman, personal communication), supporting the idea that without the thylakoid membranes, *in vitro* phosphorylation of β-phycocyanin was light-independent.

In vitro phosphorylation did not significantly chage the absorption spectra of β-phycocyanin, indicating that β-phycocyanin could still absorb certain spectral qualituies of light (maximum absorption at 615 nm to 620 nm). However, *in vitro* phosphorylation of β-phycocyanin resulted in fluorescence quenching, suggesting that there was a loss of excitation energy via non-radiative dissipation. Whether the loss of the excitation energy of β-phycocyanin will transfer to PS II (for review see Allen, 1992) or to PS I (Su *et al.*, 1992) when β-phycocyanin is phosphorylated *in vivo* is currently unknown. *In vitro* protein phosphorylation showed that β-phycocyanin was phosphorylated. However, intact phycobilisomes could not be phosphorylated. Therefore, we proposed a hypothesis that there were two compartments of β-phycocyanin in the cell, one of which were integrated to the phycobilisome via linker polypeptides and the other would have been in direct contact with the thylakoid membranes due to results that the phosphorylated β-phycocyanin was found in the membrane fractions. The latter was phosphorylated and could directly transfer the excitation energy to PS I. Transfer of the excitation energy from phycobiliprotein to PS I was supported by previous work that in UV6P, a mutant that without linker polypeptide, and the "free" phycobiliproteins could directly transfer the excitation energy to PS I without via PS II (Su *et al.*, 1992)

Since the β-phycocyanin gene (*cpcB* gene) of *Synechocystis* sp. PCC 6803 has been sequenced, the amino acid sequence of β-phycocyanin of *Synechocystis* sp. PCC 6803 can be predicted (L. Anderson, personal communication), and this provides further evidence that the phosphorylated 18 kDa protein is β-phycocyanin. The N-terminal amino

acid sequence of the 9 kDa fragment of V8 protease digested 18 kDa protein and that of the β-phycocyanin deduced from the *Synechocystis* sp. PCC 6803 *cpcB* gene sequence are identical except that the first amino acid deduced from the DNA sequence of the *Synechocystis* sp. PCC 6803 *cpcB* gene was methionine rather than valine. The difference may be explained by the observation that it is very difficult to separate valine and methionine using HPLC during the process of amino acid sequenceing or that ATG codon coding for the N-terminal methionine in bacteria sometimes codes for valine instead of methionine (Dr. K. Lilley, personal communication).

Very recently a mutant of *Synechocystis* sp. PCC 6803, 4R, where the amino acid residues after the N-terminal 27 residue of β-phycocyanin were all deleted was constructed (L. Anderson, personal communication). There are three serine residues in the β-phycocyanin of the 4R mutant. This mutant (a kind gift of L. Anderson) is now being growing in this laboratory and will be used for investigating the phosphorylation site. Nevertheless, the phosphorylation site of β-phycocyanin was focused on the serine 50 because this serine was conserved in β-phycocyanin from all the cyanobacterial strains examined and is absent in α-phycocyanin (Schirmer *et al.*, 1985; 1986). Therefore, the serine 50 in β-phycocyanin has been chosen as the target for the mutagenesis. Due to shortage of time, the site-directed mutagenesis was not done by me; however, this work is now being done in this laboratory.

Chapter 6

Conclusion and Prospects

6.1. Conclusion

This thesis has been concerned with establishing some aspects of the physiological roles of protein phosphorylation in cyanobacteria, especially focusing on the relationship between protein phosphorylation and photosynthesis. Protein phosphorylation has been found and extensively studied in higher plants, in prokaryotes and in cyanobacteria. Protein kinase activities have been found in higher plant chloroplasts and cyanobacterial thylakoid membranes and their role in state-transitions has been widely accepted. The thylakoid membranes represent most of the total membranes in cyanobacteria. Consequently it was decided to study *in vitro* protein phosphorylation in the membrane fractions of the cyanobacterium *Synechocystis* sp. PCC 6803.

Several membrane proteins were found to be phosphorylated *in vitro*, especially proteins of molecular mass 14 kDa, 18 kDa, 20 kDa and 56 kDa; thus the existence of protein kinase activities in the *Synechocystis* sp. PCC 6803 total membranes was also demonstrated. However, the phosphorylation of the 14 kDa, 20 kDa and the 56 kDa proteins was inconsistent. In contrast, the 18 kDa protein was consistently phosphorylated and found in both the phycobiliprotein-containing fractions and the thylakoid membrane fractions of a sucrose density gradient. Outgrowth of the cells, stress and cellular nutrition status might all affect protein phosphorylation. However which factor(s) leading to the inconsistent phosphorylation of the 14 kDa, 20 kDa and 56 kDa protein is still unknown. For these reasons this 18 kDa protein was chosen as the target to be studied. Phosphorylation of the 14 kDa and the 18 kDa proteins was sometimes found to occur as doublet, which might result from the cellular nutrition status; however, this still needs to be determined.

The 18 kDa protein was characterised and might be a phycobiliprotein because of its strong fluorescence under UV light. The N-terminal amino acid sequence of the 18 kDa protein showed high similarity to that of β-phycocyanin from several cyanobacterial

strains; therefore, this protein was tentatively identified as β-phycocyanin. The phosphoamino acid of β-phycocyanin (the 18 kDa protein) was identified as phosphoserine. However, the particular serine(s) in β-phycocyanin that was modified has still not been determined. Protease digestion experiments showed that β-phycocyanin was not digested by trypsin, which has also been found in *Synechocystis* sp. PCC 6308 (Duke *et al.*, 1989).

Both time course experiments and the dilution experiments suggested the existence of a phosphatase. This was confirmed by *in vitro* phosphorylation of β-phycocyanin, followed by a pulse-chase reaction. *In vitro* phosphorylation and dephosphorylation of β-phycocyanin were insensitive to classical inhibitors of protein kinases and phosphatases, such as NaF, FSBA and microcystin-LR; however, they were inhibited by EDTA, suggesting that Mg^{2+} or another divalent cation was required for both the activities of β-phycocyanin kinase and the phosphatase.

Purification of the β-phycocyanin kinase suggested that β-phycocyanin, the β-phycocyanin kinase and the phosphatase were located in a macromolecular complex (the β -phycocyanin complex), but it is still unknown whether or not the β-phycocyanin kinase and the phosphatase are either the different domains of the same enzyme molecule such as seen with isocitrate dehydrogenase kinase/phosphatase (Nimmo *et al.*, 1984) or different proteins. The β-phycocyanin kinase was a stable enzyme and its activity was not affected by reagents used for preparation of the thylakoid membranes and isolation of the phycobilisomes. The β-phycocyanin kinase lost its activity in urea, but the activities of the β-phycocyanin kinase and the phosphatase could be restored when urea was diluted. However, the β-phycocyanin kinase activity could not be reconstituted when the β-phycocyanin complex was subjected to anion exchange chromatography in the presence of urea. The possibility that β-phycocyanin could not be phosphorylated after reconstitution might be either the loss of molecular chaperone or the stoichiometry of the β-phycocyanin complex being incorrectly matched. Evidence has been shown that EDTA will inhibit the

formation of protein aggregates, as divalent cations, especially Ca^{2+}, is required for proteins to form aggregates. EDTA also inhibited phosphorylation of β-phycocyanin; therefore, it could also be possible that β-phycocyanin, β-phycocyanin kinase and the other proteins occurred in a protein aggregate. β-phycocyanin kinase will express its activity when it occurred in the aggregate; on the contrary, β-phycocyanin lost its activity when the protein aggregate was destroyed. The β-phycocyanin kinase could not phosphorylate either casein or histone, suggesting that the β-phycocyanin kinase was probably specific for β-phycocyanin.

Phosphorylation of β-phycocyanin was found to be light-independent *in vitro*., which was inconsistent with that found in *Synechococcus* sp. PCC 6301 (Harrison *et al*., 1991). The apparent discrepancy might be because of the presence of the thylakoid membranes (Allen *et al*., 1981; Allen and Horton, 1981; Gal *et al*., 1988). Phosphorylation of β-phycocyanin did not affect the absorption spectra of β-phycocyanin; however, phosphorylation of β-phycocyanin resulted in fluorescence quenching, suggesting loss of the excitation energy via non-radiative dissipation. *In vitro* protein phosphorylation showed that intact phycobilisomes could not be phosphorylated. Therefore it could possibly be that there were two components of β-phycocyanin. One may be integrated into phycobilisomes via a linker polypeptide and the other may be in direct contact with the thylakoid membranes. When the latter was phosphorylated it might directly transfer the excitation energy to PS I.

The 18 kDa protein was tentatively identified β-phycocyanin. In order to confirm this, cloning and sequencing the *Synechocystis* sp. PCC 6803 *cpcB* gene, encoding β-phycocyanin were performed. An expected size of 0.5 kb PCR product of the *Synechocystis* sp. PCC 6803 *cpcB* gene was found. A cosmid clone was also found to contain the *Synechocystis* sp. PCC 6803 *cpcB* gene. Recently, the *Synechocystis* sp. PCC 6803 *cpcB* gene was cloned and sequenced (L. Anderson, personal communication) and

the predicted amino acid sequence provided further evidence that the 18 kDa protein was β-phycocyanin.

6.2. Prospects

Site-directed mutations preventing β-phycocyanin phosphorylation can be created, thereby providing more information about the localisation of the phosphorylation site and the physiological function(s) of the phosphorylation of β-phycocyanin.

Phosphorylation of β-phycocyanin *in vitro* resulted in fluorescence quenching, suggesting that there was excitation energy loss via non-radiative dissipation. Therefore, it would be very interesting to know the redistribution of excitation energy when β-phycocyanin is phosphorylated *in vivo*.

The β-phycocyanin kinase and the phosphatase co-occur in the β-phycocyanin complex and could not be separated. Therefore, it is of interest to know whether the β-phycocyanin kinase and the phosphatase are located in the different domains of the same enzyme molecule or they are different subunits of the β-phycocyanin complex. Recently, a Japanese group has started sequencing the whole genome of *Synechocystis* sp. PCC 6803 and more than a quarter of the genome has already been sequenced and released to the scientific field on Cyanobase Homepage (Dr. H. Kotani; E-URL: http://www.kazusa.or.jp/cyano/cyano.html). Therefore, the β-phycocyanin kinase gene may be known in the near future and furthermore more detail information about the β-phycocyanin kinase will be understood.

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